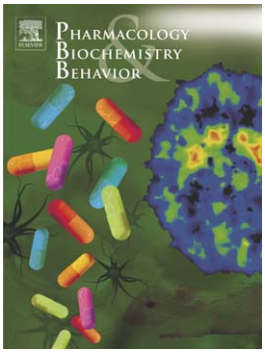

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R-phenibut binds to the α_2-δ subunit of voltage-dependent calcium channels and exerts gabapentin-like anti-nociceptive effects

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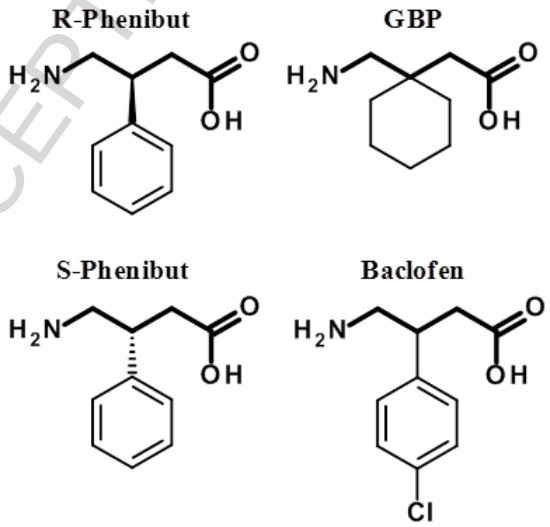
Abstract

Phenibut is clinically used anxiolytic, mood elevator and nootropic drug. R-phenibut is responsible for the pharmacological activity of racemic phenibut, and this activity correlates with its binding affinity for $GABA_B$ receptors. In contrast, S-phenibut does not bind to $GABA_B$ receptors. In this study, we assessed the binding affinities of R-phenibut, S-phenibut, baclofen and gabapentin (GBP) for the α_2-δ subunit of the voltage-dependent calcium channel (VDCC) using a subunit-selective ligand, radiolabelled GBP. Binding experiments using rat brain membrane preparations revealed that the equilibrium dissociation constants (K_is) for R-phenibut, S-phenibut, baclofen and GBP were 23, 39, 156 and 0.05 μM, respectively. In the pentylenetetrazole (PTZ)-induced seizure test, we found that at doses up to 100 mg/kg, R-phenibut did not affect PTZ-induced seizures. The anti-nociceptive effects of R-phenibut were assessed using the formalin-induced paw-licking test and the chronic constriction injury (CCI) of the sciatic nerve model. Pre-treatment with R-phenibut dose-dependently decreased the nociceptive response during both phases of the test. The anti-nociceptive effects of R-phenibut in the formalin-induced paw-licking test were not blocked by the $GABA_B$ receptor-selective antagonist CGP35348. In addition, treatment with R- and S-phenibut alleviated the mechanical and thermal allodynia induced by CCI of the sciatic nerve. Our data suggest that the binding affinity of R-phenibut for the α_2-δ subunit of the VDCC is 4 times higher than its affinity for the $GABA_B$ receptor. The anti-nociceptive effects of R-phenibut observed in the tests of formalin-induced paw licking and CCI of the sciatic nerve were associated with its effect on the α_2-δ subunit of the VDCC rather than with its effects on $GABA_B$ receptors. In conclusion, our results provide experimental evidence for GBP-like, anti-nociceptive properties of R-phenibut, which might be used clinically to treat neuropathic pain disorders.

Introduction

R-phenibut ((3R)-phenyl-4-aminobutyric acid) is the optically pure and pharmacologically active form of racemic phenibut, a derivative of γ-aminobutyric acid (GABA) (Dambrova et al., 2008). Compared to the penetration of GABA, the penetration of phenibut through the blood-brain barrier is significantly improved due to the incorporation of a phenyl ring into the GABA molecule (Lapin et al., 2001). Structurally, R-phenibut is related to baclofen ((RS)-4-amino-3-(4-chlorophenyl)butanoic acid) and gabapentin (GBP, (2-[1-(aminomethyl)cyclohexyl]acetic acid)), drugs that mimic the chemical structure of the neurotransmitter GABA. Another optical isomer of racemic phenibut is S-phenibut, which does not bind to $GABA_B$ receptors (Dambrova et al., 2008). Baclofen is a $GABA_B$ receptor-active compound; however, GBP does not bind to these receptors but exerts its anti-nociceptive and anti-convulsant effects by binding to the α_2-δ subunit of the voltage-dependent calcium channel (VDCC). As shown in Figure 1, R-phenibut is structurally related to substances that mimic the chemical structure of the neurotransmitter GABA: S-phenibut, baclofen and GBP.

Fig. 1. Chemical structures of R-phenibut, S-phenibut, baclofen and GBP.



Baclofen acts on metabotropic $GABA_B$ receptors (Bowery et al., 2006). In clinical practice, baclofen has been used to treat muscle spasticity and chronic cough (Bowery, 2007); in addition, clinical trials have shown that baclofen can be used to treat alcohol dependence (Addolorato et al., 2007). However, clinical use of baclofen is frequently associated with undesired side effects, such as drowsiness, dizziness, muscle weakness, mental confusion and sexual dysfunction (Bowery, 2006; Calabrò et al., 2014). Our

previous study has unambiguously shown that both baclofen and R-phenibut bind directly to the $GABA_B$ receptor, although the affinity of baclofen is approximately 15 times higher than that of R-phenibut (Dambrova et al., 2008). Like baclofen, R-phenibut has also been shown to exhibit anti-nociceptive effects in acute pain models, such as the tail-flick and hot-plate tests in rodents, and these effects of R-phenibut can be completely blocked by the $GABA_B$ receptor antagonist CGP35348 (Aley and Kulkarni, 1991; Dambrova et al., 2008). However, S-phenibut does not exhibit any anti-depressant, anti-nociceptive or locomotor effects and does not bind to $GABA_B$ receptors (Dambrova et al., 2008). Despite the similar mechanisms of action of phenibut and baclofen at the $GABA_B$ receptor, phenibut is used for different indications in clinical practice. Phenibut is primarily used as a mood elevator and tranquilizer as well as a pre- and/or post-operative medication (Lapin, 2001). Due to its high anxiolytic and cognition-enhancing effects, it has been included in medical kits for space flights (Neumyvakin et al., 1978). The side effects of the clinical use of phenibut are less pronounced than those of baclofen, which include irritability, agitation, dizziness, headache, and drowsiness. At very high concentrations, baclofen is known to displace [^{3}H]GBP in rat synaptic plasma membranes (Suman-Chauhan et al., 1993).

GBP binding sites are heterogeneously distributed in rat brain tissues (Hill et al., 1993; Bian et al., 2004). Gee et al. have purified and characterized a high-affinity [^{3}H]GBP-binding protein from pig brain membranes, and N-terminal sequencing identified the protein as the α_2-δ subunit of the VDCC (Gee et al., 1996). This conclusion was supported by tissue distribution studies, hydrodynamic data, heterologous expression of cloned α_2-δ cDNA in COS-7 and HEK cells, and by radioligand binding and immunoblotting studies of fractionated Ca^{2+} channel subunits. [^{3}H]GBP was the first ligand described as interacting with the α_2-δ subunit (Gee et al., 1996). Subsequently, GBP was shown to bind to the α_2-δ_1 and α_2-δ_2 subunits with high affinity (K_d=59 and 153 nM, respectively), but it does not bind to the α_2-δ_3 and α_2-δ_4 subunits (Marais et al., 2001; Qin et al., 2002). Although GBP is a structural analogue of GABA, binding of GBP to $GABA_A$ or $GABA_B$ receptors was not observed. For example, in concentrations up to 1 mM, GBP did not displace [^{3}H]GABA from $GABA_B$ receptor sites in rat synaptic membranes (Jensen et al., 2002). In addition to being an anti-convulsive drug, GBP is now considered to be an effective drug for some forms of neuropathic and post-surgical pain (O'Connor and Dworkin, 2009; Zhang et al., 2013; Victoria et al., 2008; Caraceni et

al., 1999). Field et al. have shown that the anti-nociceptive effects of GBP and pregabalin result from their binding to the α_2-δ_1 subunits of VDCCs. In addition, spontaneous and induced mutations that affect the α_2-δ_2 gene have been shown to be associated with epilepsy, enhanced seizure susceptibility and ataxia (Barclay et al., 2001). Moreover, Cacna2d2 null mice show increased susceptibility to seizures induced by the convulsant pentylenetetrazole (PTZ; Ivanov et al., 2004), indicating that the anti-convulsant activity of GBP is associated with binding to the α_2-δ_2 subunit of the VDCC.

In the current study, we tested the binding affinity of R- and S-phenibut to the α_2-δ subunit of the VDCC in rat brain membranes using a subunit-selective ligand, radiolabelled GBP. To study the potentials of these compounds to alleviate neuropathic pain, we used experimental pain models of formalin-induced paw licking and chronic constriction injury (CCI) of the sciatic nerve. To delineate the mechanism of the anti-nociceptive effects of R-phenibut in the neuropathic pain models, we used an antagonist of $GABA_B$ receptors, CGP35348 (Ople et al., 1990), and S-phenibut, a structurally related compound that does not bind to $GABA_B$ receptors (Dambrova et al., 2008).

Materials and methods

2.1. Chemicals

R-phenibut was obtained from JSC Olainfarm (Olaine, Latvia). Racemic baclofen was from Polpharma, Poland. GBP was obtained from TCI Europe nv (Zwijndrecht, Belgium). [^{3}H]Gabapentin (1-(Amino-[^{3}H]-methyl)-[2,3,5,6-^{3}H]-cyclohexylacetic acid; specific activity 110 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, Missouri, USA). 3-Aminopropyl(diethoxymethyl)phosphinic acid (CGP35348) was obtained from Tocris Bioscience (Bristol, United Kingdom).

Animals

Male ICR mice and Wistar rats (Laboratory Animal Breeding Facility, Riga Stradins University, Latvia), weighing 23-25 g and 350-400 g, respectively, were housed under standard conditions (21-23°C, 12-h light-dark cycle) with unlimited access to standard food (Lactamin AB, Sweden) and water. All experimental procedures were carried out in accordance with the guidelines of the EU Directive 2010/63/EU and with local laws and policies and were approved by the Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

2.2. [^{3}H]GBP binding assay

Membranes were prepared as described previously [Sumam-Chauhan et al., 1993] with slight modifications. Briefly, male Wistar rats were decapitated, and the brains were dissected. The cortex was rapidly homogenized in 10 volumes (ratio of grams of wet brain tissue to mL of buffer) of Buffer A (5 mM Tris and 5 mM EDTA, pH=7.4 at 4°C) containing 0.32 M sucrose using a glass homogenizer. The homogenates were centrifuged at 950 × g for 10 min at 4°C (Heraeus™ Biofuge™ Stratos™ Centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The supernatants were then centrifuged at 50000 × g for 30 min at 4°C in an Optima™ L-100 XP ultracentrifuge (Beckman Coulter International SA, Nyon, Switzerland). The resulting pellets were re-suspended in 20 volumes (with respect to the original wet weight) of Buffer B (5 mM Tris and 5 mM EDTA, pH=8.0 at 4°C) and stirred on ice for 30 min. The suspensions were then centrifuged at 50000 × g for 30 min at 4°C. The pellets were re-suspended in Buffer A

containing 1.2 M sucrose and further separated on sucrose gradients (Buffer B containing 0.0, 0.9 or 1.2 M sucrose; 1:1:1, vol/vol/vol) by centrifugation with a 90Ti rotor at 100000 × g for 90 min. The synaptic plasma membranes located at the interface between 0.9 and 1.2 M sucrose were collected, resuspended in 2 mL Buffer A, and centrifuged at 50000 × g for 30 min. The final pellet was re-suspended in Buffer A and frozen in aliquots (protein concentration of 6-8 mg/mL) at -80°C until use. Protein concentrations were measured by the Lowry method [Lowry et al., 1951] with some modifications, using bovine serum albumin as the standard. On the day of use, the membrane aliquots were thawed, centrifuged at 50000 × g for 30 min and then re-suspended in incubation buffer (10 mM HEPES, pH=7.4 at 4°C). The binding assay buffer consisted of 110 µL of incubation buffer, 50 µL of membrane aliquots, either 20 µL of the tested drug or deionized water for the control, and 20 µL of [^{3}H]GBP. The final concentration of [3H]GBP was 1.0 nM. Nonspecific binding was assessed by adding GBP (100 µM). The samples were incubated for 40 min at room temperature. The bound and free radioligands were separated by rapid filtration under a vacuum using Millipore GF/B filter paper (Merck Millipore, Billerica, Massachusetts, USA). The filters were washed three times with 0.25 mL of 50 mM Tris (pH 7.4 at 4°C). The samples' radioactivities were measured with a liquid scintillation counter (Wallac MicroBeta TriLux (PerkinElmer, Waltham, Massachusetts, USA)). Each experiment was repeated at least three times, and each assay was conducted in duplicate.

2.3. Behavioural tests

2.3.1. Formalin-induced paw-licking test

The formalin-induced paw-licking test was performed as described previously (Zvejniece et al., 2006). All of the animals received drug or saline 60 min prior to intraplantar injection of formalin. GBP (50 mg/kg) and R-phenibut (25, 50 and 100 mg/kg) were administered intraperitoneally (i.p.). The control mice received an i.p. injection of saline. The mice were gently restrained, and 30 µL of formalin solution (1.5% in saline) was injected subcutaneously (s.c.) into the plantar surface of the right hind paw using a microsyringe with a 27-gauge needle. Each mouse was then placed in an individual clear Plexiglas observation chamber (30×20×30 cm). For each mouse, the total time spent licking the hind paw was recorded and quantified in 5-min intervals over 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.

For the antagonism studies, the mice received an i.p. injection of CGP35348 at a dose of 100 mg/kg 10 min prior to the administration of R-phenibut or GBP at a dose of 50 mg/kg. R-phenibut or GBP was administered at 50 min prior to the experiment. The dose of CGP35348 used was based on previous experiments, in which CGP35348 was found to significantly block the pharmacological activity of R-phenibut at a dose of 100 mg/kg (Dambrova et al., 2008).

2.3.2. Thermal and mechanical allodynia after CCI of the sciatic nerve

2.3.2.1. Surgical procedures and drug administration

Persistent neuropathic pain was evoked in rats by CCI of the sciatic nerve according to the model of Bennett and Xie (1988). Briefly, anaesthesia was induced with 5% isoflurane in a mixture of 50% nitrous oxide and 50% oxygen and maintained with 2% isoflurane using a face mask. The anaesthetized animals received s.c. administration of tramadol (5 mg/kg) to attenuate acute pain. The left sciatic nerve was exposed by blunt dissection through the biceps femoris, and four ligatures (cotton thread) were loosely tied, 1 mm apart, just proximal to the trifurcation of the sciatic nerve. In sham-operated animals, the left sciatic nerve was exposed in the same manner but was not ligated.

The rats were divided into the following six experimental groups: sham-operated rats (n=8), CCI control rats (n=8), CCI rats that received a 25 mg/kg dose of R-phenibut (n=8), CCI rats that received a 50 mg/kg dose of R-phenibut (n=8), CCI rats that received a 50 mg/kg dose of S-phenibut (n=8) and CCI rats that received a 100 mg/kg dose of GBP (n=8).

The behavioural tests were performed before surgery and on post-operative days 6 (baseline) and 7 (experimental day). R- and S-phenibut and GBP were administered i.p., and then their effects on thermal and mechanical allodynia were examined at 1 and 4 h post-injection on the 7^{th} day after the operation.

2.3.2.2. Electronic von Frey test

Mechanical allodynia was assessed in the CCI rats by measuring the withdrawal threshold of the left hind paw 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). During the tests, the rats 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 each test session. The von Frey filament was applied to the midplantar surface of the left hind paw. The withdrawal threshold was defined as the average force (g) required to cause withdrawal of the stimulated paw over five trials.

2.3.2.3. Cold plate test

Thermal allodynia was assessed in the CCI rats using the cold plate test (Hot/Cold Plate, model 35100, Ugo Basile, Varese, Italy). The rats were placed on a cold stainless steel plate maintained at 3°C, and the withdrawal latency, with respect to licking of the hind paw, was recorded in seconds. The cut-off latency was 90 s.

2.3.3. PTZ-induced seizures

To more accurately detect even slight modulatory effects on convulsive tendencies, we employed the more sensitive intravenous (i.v.) administration route for PTZ administration (Loscher et al., 1991; Mandhane et al., 2007; Zvejniece *et al*., 2010). PTZ-induced clonic and tonic seizures were initiated by inserting a 30-gauge needle into the tail vein of restrained mice and infusing a 1% solution of PTZ at a constant rate of 20 μL/2 s. The infusion was halted when forelimb clonus followed by tonic seizures of the full body were observed. The minimal doses of PTZ (mg/kg of mouse weight) necessary to induce clonic and tonic seizures were considered as an index of seizure threshold.

Thirty minutes prior to PTZ administration, R-phenibut and GBP were injected at doses of 50 and 100 mg/kg, while baclofen was administered at a dose of 2.5 mg/kg i.p.

2.4. Statistical analysis

All results were expressed as the mean ± S.E.M. The effects of R-phenibut on the duration of nociceptive behaviours during the first and second phases of the formalin test and on anti-convulsant activity in the PTZ-induced seizure test were analysed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons tests. The behaviour after CCI of the sciatic nerve was evaluated by two-way repeated-measures ANOVA and one-way ANOVA followed by Newman-Keuls multiple comparisons tests and Student's t-tests. Statistical calculations were performed using GraphPad Prism 3.0 software package (GraphPad Software, Inc., La Jolla, California, USA). P-values of less than 0.05 were considered statistically significant. The potencies of the tested drugs for displacing the radioligand from the receptors were also calculated using GraphPad Prism 3.0 software package.

3. Results and Discussion

3.1. [^{3}H]GBP binding assay

R- and S-phenibut and baclofen (1 nM to 1 mM) were tested for their binding affinities to the α_2-δ subunit of the VDCC. The drugs interacted with the [^{3}H]GBP-defined site in a competitive fashion. R- and S-phenibut and baclofen demonstrated affinities for the α_2-δ subunit of the VDCC, with calculated K_i values of 23±6 μM, 39±5 μM and 156±40 μM, respectively (Fig. 2, Table 1). Non-labelled GBP was used as a reference drug, for which a characteristic competition curve with an average K_i value of 0.05±0.01 μM was obtained (Fig. 2).

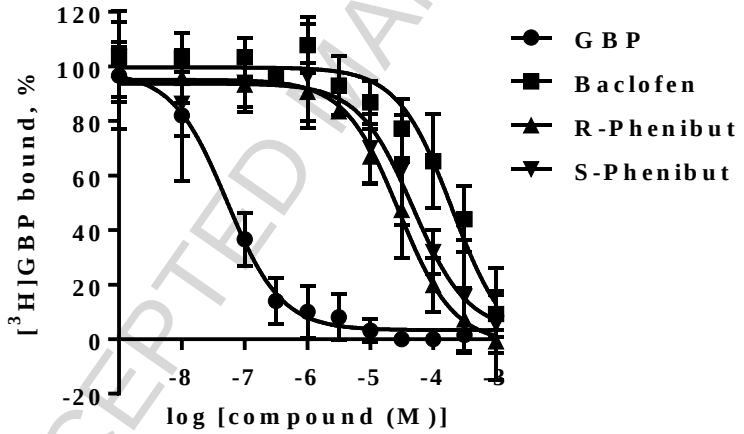


Fig. 2. The effects of R-phenibut (▲), S-phenibut (▼), baclofen (■) and GBP (●) on the binding of [^{3}H]GBP to the α_2-δ subunit of the VDCC in membranes prepared from male Wistar rat brains. The data represent at least three experiments performed in duplicate.

GBP exerts its pharmacological activity through binding to the α_2-δ subunit of the VDCC, but it does not bind to $GABA_B$ receptors. R-phenibut interacted with the α_2-δ subunit of the VDCC in a competitive fashion, and its affinity was 7-fold higher than that of baclofen (Fig. 2). Moreover, the binding of S-phenibut to the α_2-δ subunit of the VDCC was only slightly lower than that of R-phenibut (Table 1). In addition, we have previously shown that R-phenibut binds directly to the $GABA_B$ receptor and that it is approximately 15 times less active than baclofen (Dambrova et al., 2008). Taken together, these results provide the first demonstration that the pharmacological effects of R-phenibut are mediated through both the α_2-δ subunit of the VDCC and $GABA_B$

receptors, whereas S-phenibut binds selectively to the α_2-δ subunit of the VDCC. The binding affinity of R-phenibut for the α_2-δ subunit of the VDCC is 4 times higher than its affinity for the $GABA_B$ receptor (Table 1). As shown in the Table, R-phenibut exhibits a higher affinity than baclofen for the α_2-δ subunit of the VDCC.

Table 1

Equilibrium dissociation constants of R-phenibut, S-phenibut, GBP and baclofen for the α_2-δ subunit of the VDCC and $GABA_B$ receptor.

	α_2-δ subunit of the VDCC Ki (μM)	$GABA_B$ Ki (μM)
R-phenibut	23 ± 6	92 ± 3[a]
S-phenibut	39 ± 5	> 1 mM [a]
Baclofen	156 ± 40	6 ± 1[a]
GBP	0.05 ± 0.01	> 1 mM [b]

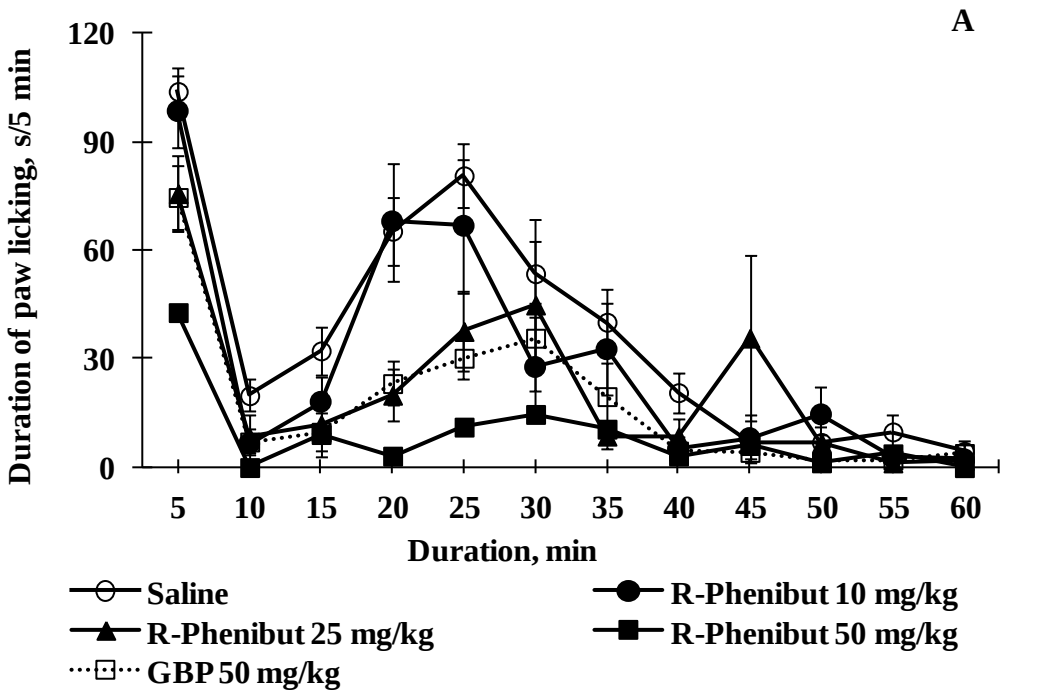
[a] Dambrova et al., 2008; [b] Jensen et al., 2002.

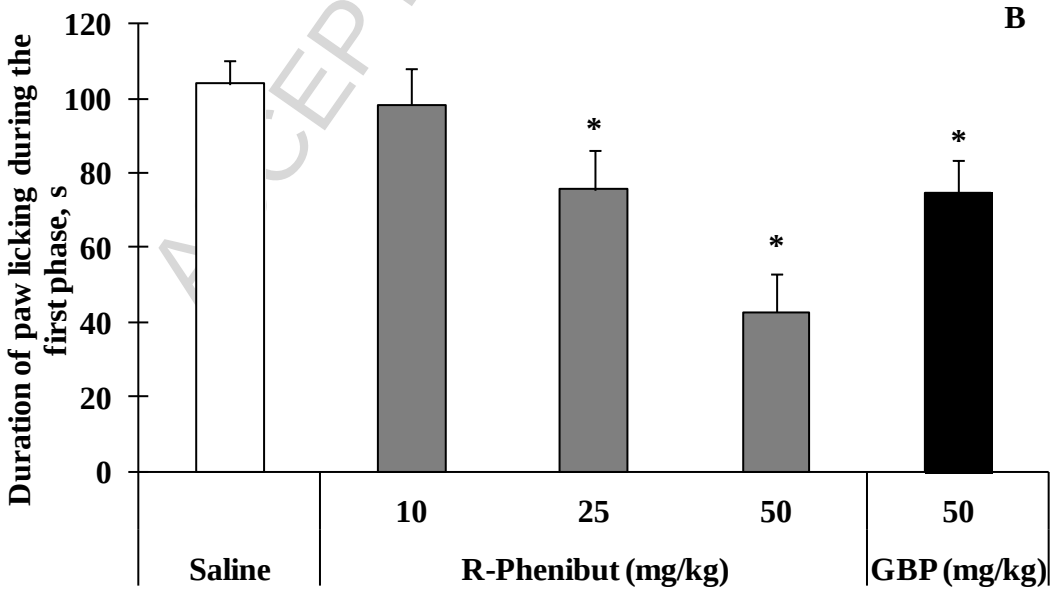
3.2. Neuropathic pain models

3.2.1. Formalin-induced paw-licking test

The anti-nociceptive effects of R-phenibut were studied in the formalin-induced paw-licking test in mice (Fig. 3A). As shown in Figure 3B, 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 formalin injection in the saline-treated mice and lasted for an additional 35–40 min (Fig. 3C). As shown in Figure 3B, R-phenibut (10, 25 and 50 mg/kg, i.p.) dose-dependently reduced the formalin-induced nociceptive behaviour during the first phase ($F_{(4, 79)}$=6.958, p<0.0001). During this phase, 25 and 50 mg/kg R-phenibut significantly reduced the duration of paw licking by approximately 30 (p<0.05) and 60% (p<0.001), respectively. A similar effect of R-phenibut was observed during the second phase ($F_{(4, 79)}$=10.14, p<0.0001), in which it significantly reduced paw-licking time by approximately 55% and 85% at doses of 25 and 50 mg/kg, respectively (Fig. 3C). At a dose of 50 mg/kg, GBP significantly reduced

paw-licking time by approximately 32% during the first phase (p<0.05) and 56% during the second phase (Fig. 3B, C, p<0.001).





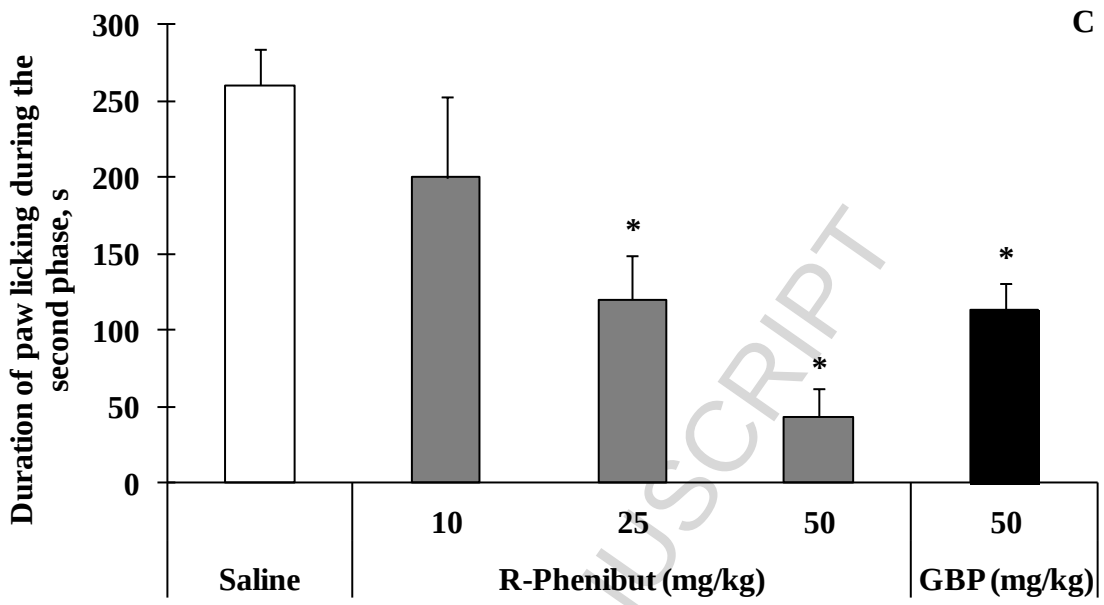
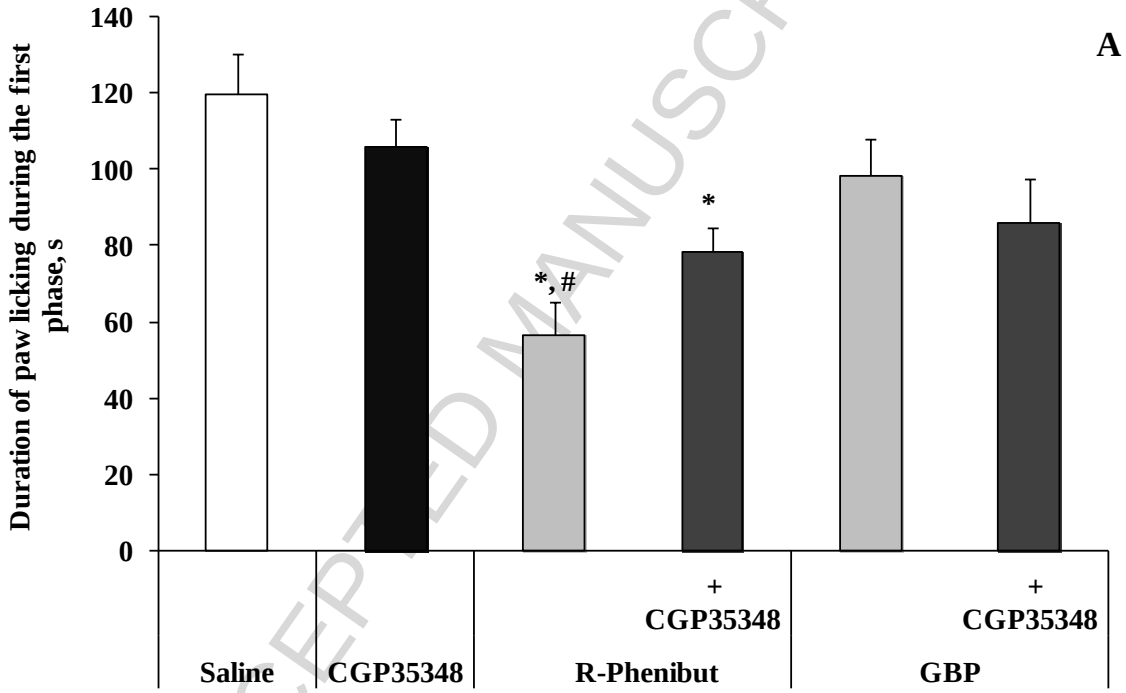


Fig. 3. Dose-dependent effects of R-phenibut on anti-nociceptive behaviours in the formalin-induced paw-licking test in mice. R-phenibut (10, 25 and 50 mg/kg) and GBP (50 mg/kg) were administered i.p. at 30 min before the test. Formalin (1.5%; 30 µL) was injected s.c. into the plantar surfaces of the 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. **(B)** The first phase was from 0 to 5 min and the second phase was from 15 to 40 min **(C)** after injection of formalin. The data are expressed as the mean±SEM (n=9–15) and were analysed by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. $^{*}p<0.05$ vs. the saline group.

The $GABA_B$ receptor antagonist CGP35348 did not alter nociceptive behaviour in the formalin-induced paw-licking test during the first or second phase (Fig. 4A, B). During the first and second phase, R-phenibut (50 mg/kg) significantly reduced paw-licking time compared to saline and CGP35348 ($F_{(5, 52)}$=4.677, p<0.001). Pre-treatment with GBP also reduced the nociceptive response in the formalin-induced paw-licking test; however, during the first phase, the effect was not significant (Fig. 4A). As shown in Figure 4B, co-administration with CGP35348 did not affect the anti-nociceptive effects of R-phenibut and GBP during the second phase of the formalin-induced paw-licking test, and the licking behaviours of these groups remained significantly different from those of the saline- and CGP35348-treated groups (Fig. 4B, $F_{(5, 52)}$=8.309, p<0.001). As shown in

Figure 4A, the anti-nociceptive response during the first phase of the test remained significantly reduced in the CGP35348 and R-phenibut combination group compared with the saline-treated group ($p<0.05$), but the licking behaviour of this group did not differ from those of the CGP35348-treated groups. Pre-treatment with CGP35348 slightly reduced the anti-nociceptive effect of R-phenibut by approximately 30% during the first phase, but the effect was not statistically significant.



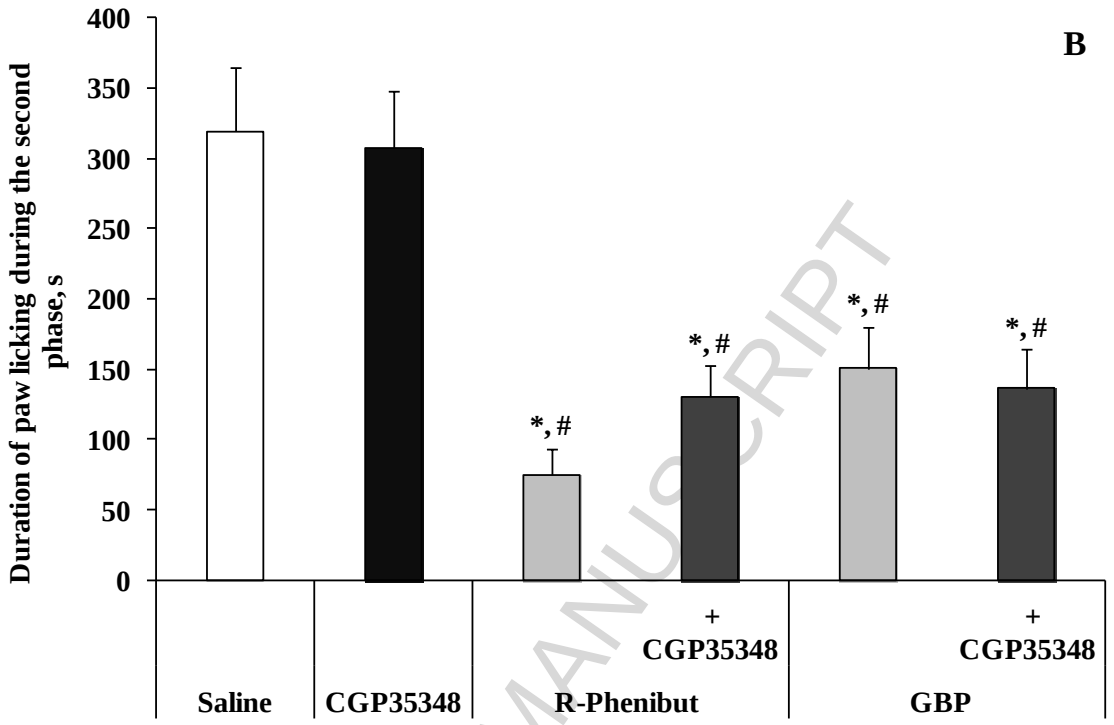


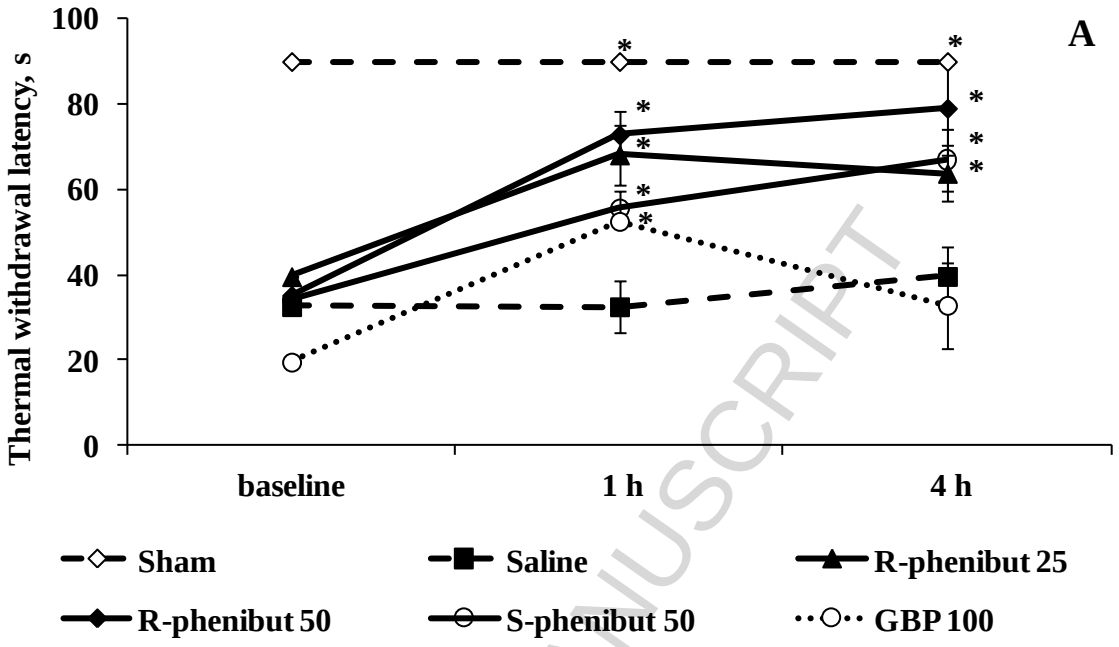
Fig. 4. The effect of 100 mg/kg CGP35348 on the anti-nociceptive effects of R-phenibut and GBP in the formalin-induced paw-licking test in mice. R-phenibut (50 mg/kg) and GBP (50 mg/kg) were administered i.p. at 50 min before the test, and CGP35348 (100 mg/kg) was administered i.p. at 10 min before R-phenibut or GBP administration. Formalin (1.5%; 30 μL) was injected s.c. into the plantar surfaces of the right hind paws of the mice. Licking of the injected paw was recorded as the total time (s) spent licking within each of twelve 5-min bins for a total duration of 60 min. **(A)** The first phase was from 0 to 5 min, and **(B)** the second phase was from 15 to 40 min after injection of formalin. The data are expressed as the mean±SEM (n=6–12) and were analysed by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. $^{*}p<0.05$ vs. the saline group; $^{\#}p<0.05$ vs. the CGP35348 group.

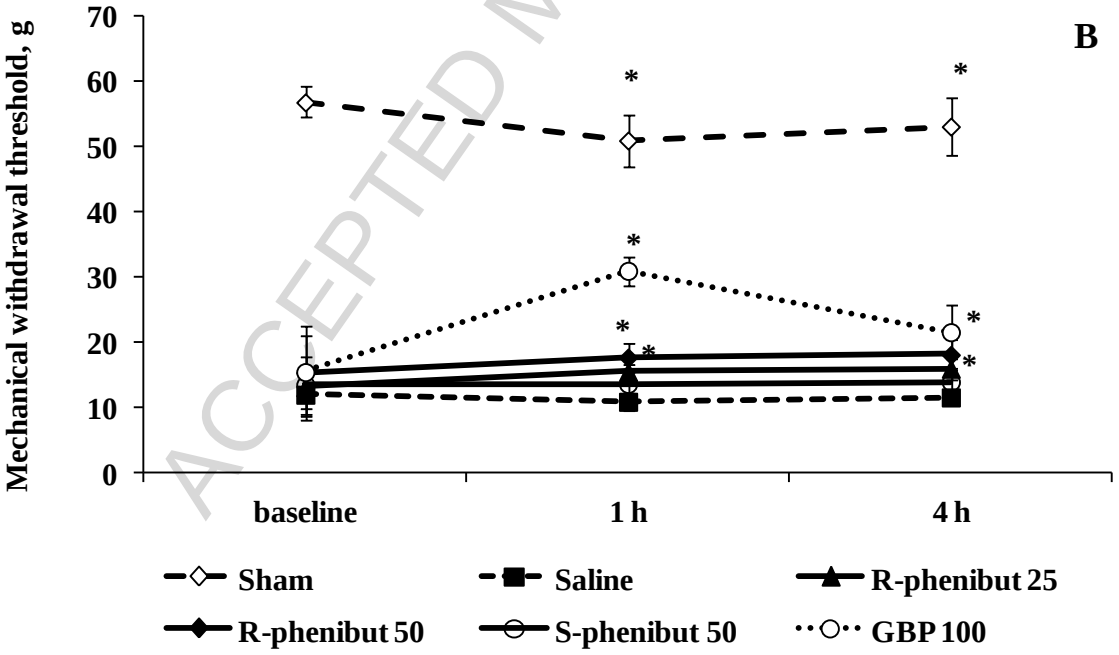
3.2.2. Thermal and mechanical allodynia after CCI of the sciatic nerve

The CCI-operated animals demonstrated clear thermal allodynia during the post-operative day 6 cold plate test. None of the sham-operated rats displayed lifting or shaking of the CCI hind paw before the 90-s cut off, whereas the baseline withdrawal latency after CCI was 32.2±3.4 s, which was significantly less than that of the sham-operated group (Fig.

5A, $p<0.0001$). Two-way repeated-measures ANOVA showed that the compounds attenuated the CCI-induced cold allodynia in the rats (Fig. 5A, main effect of time ($F_{(3, 42)}=35.63$, $p<0.0001$) and group ($F_{(5, 126)}=8.60$, $p<0.0001$) and interaction between group and time ($F_{(15, 126)}=14.57$, $p<0.0001$). Compared to saline, 25 and 50 mg/kg R-phenibut significantly increased the withdrawal latencies in the cold plate test at 1 and 4 h post-injection ($p<0.001$). In addition, at a dose of 50 mg/kg, S-phenibut significantly attenuated thermal allodynia at 1 and 4 h post-injection compared to saline (Fig. 5A, $p<0.001$). Compared to saline, the injection of 100 mg/kg GBP significantly increased the withdrawal latencies in the cold plate test at 1 h post-injection ($p<0.05$) but not after 4 h (Fig. 5A).

After CCI of the sciatic nerve, the animals showed mechanical allodynia during the electronic von Frey test at 6 days after surgery (Fig. 5B). The mean withdrawal threshold to mechanical stimulation of the operated rats was significantly less than that of the sham-operated rats (14.0±0.7 g and 56.8±3.9 g, respectively, $p<0.0001$, Fig. 5B). Two-way repeated-measures ANOVA showed that the compounds time-dependently attenuated the CCI-induced mechanical allodynia in the rats (Fig. 5B, C, main effects of time ($F_{(3, 43)}=261.0$, $p<0.0001$) and group ($F_{(5, 129)}=46.68$, $p<0.0001$) and interaction between group and time ($F_{(15, 129)}=12.97$, $p<0.0001$)). At doses of 25 and 50 mg/kg, R-phenibut significantly increased the withdrawal threshold at 1 and 4 h post-administration by 40% and 60%, respectively, compared with saline (Fig. 5B, $p<0.05$). After S-phenibut administration at a dose of 50 mg/kg, the withdrawal threshold of the left hind paw in response to mechanical stimulus was increased (20%), but the effect did not significantly differ from that of saline (Fig. 5B). Compared with saline, GBP significantly increased the withdrawal threshold at 1 and 4 h after administration ($p<0.01$). As demonstrated by the data expressed as areas under the curve (AUCs) (Fig. 5C), R-phenibut and GBP significantly attenuated mechanical allodynia ($F_{(5,48)}=62.98$, $p<0.0001$) in the CCI rats.





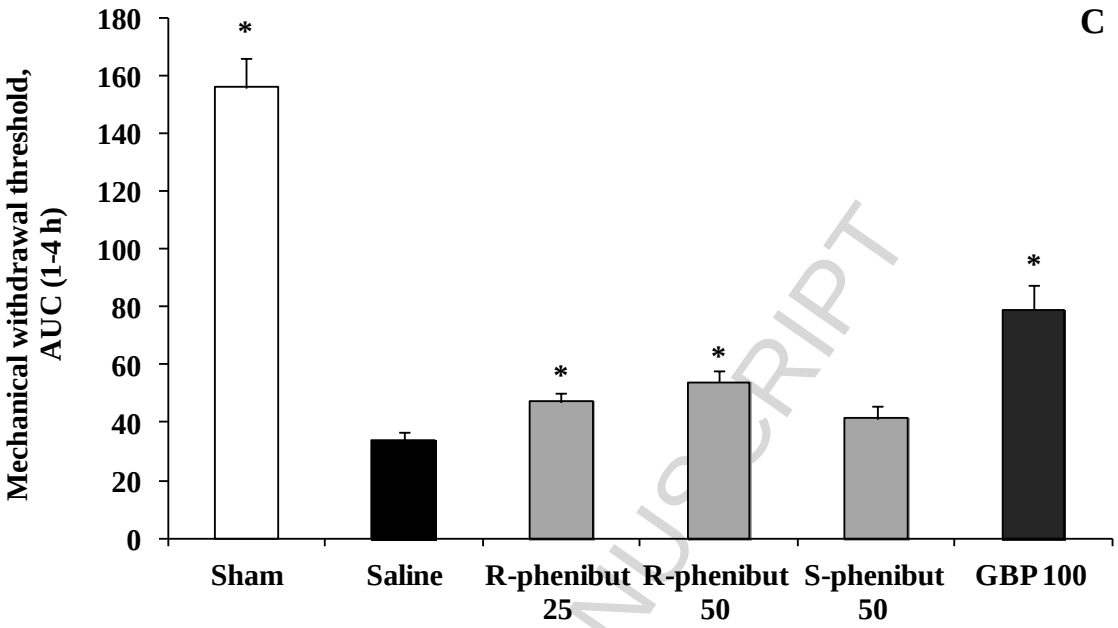


Fig. 5. Effects of R- and S- phenibut on cold and mechanical allodynia in a CCI model in rats. Cold and mechanical allodynia were measured on the 6th day (baseline) after CCI and at 1 h and 4 h after drug administration on post-operative day 7. R-phenibut (25 and 50 mg/kg), S-phenibut (50 mg/kg) and GBP (100 mg/kg) were administered i.p. (**A**) Thermal allodynia was assessed in the CCI rats using the cold plate test. The data are expressed as the latency in seconds. (**B**) Mechanical allodynia was assessed in the CCI rats using the electronic von Frey test. The data are expressed as the threshold in grams. (**C**) Areas under the curve (AUCs) were calculated from 1 h to 4 h after drug administration in the electronic von Frey test. The data are expressed as the mean±SEM (n=8) and were analysed by two-way and one-way ANOVA followed by Newman-Keuls multiple comparisons tests and Student's t-tests. *p<0.05 vs. the saline group.

The anti-nociceptive effects of GBP in neuropathic pain models are mediated through the α_2-δ_1 subunits of VDCCs (Field et al., 2007). Our results provide evidence that R-phenibut also exerts dose-dependent analgesic effects during both phases of the formalin test and that this activity is similar to the effect induced by GBP (Fig. 2 A, B, C). In the present study, administration of R-phenibut significantly attenuated CCI-induced behavioural alterations, including cold and mechanical allodynia (Fig. 5A, B, C). Our previous study has shown that the anti-nociceptive (hot plate test), locomotor (open field test) and antidepressant effects (forced swim test) of R-phenibut are blocked by the

$GABA_B$ receptor antagonist CGP35348 (Dambrova et al., 2008). In the present study, we observed that during the second phase of the formalin test, CGP35348 did not inhibit the analgesic activity of R-phenibut. Because there are no pharmacological antagonists that can block the effect of GBP mediated through the α_2-δ_1 subunits of VDCCs, we used S-phenibut, a $GABA_B$ receptor-inactive enantiomer of phenibut (Dambrova et al., 2008), to isolate the contribution of $GABA_B$ receptor-related activity to the analgesic effects of R-phenibut. The finding that S-phenibut also attenuated CCI-induced cold allodynia (Fig. 5 A) strengthens the conclusion that the anti-nociceptive activity of R-phenibut in neuropathic pain models is associated with its effect on the α_2-δ subunit of VDCCs rather than with its activity at $GABA_B$ receptors.

At a dose of 1.5 mg/kg, baclofen also exhibits analgesic effects during the second phase of the formalin-induced paw-licking test (Akada et al., 2005). However, baclofen treatment (2.5-5 mg/kg range) induces pronounced side effects, including sedation (Li at al., 2013), muscle relaxation (Akada et al., 2005; Cryan et al., 2004) and decreased locomotor activity (Farkas at al., 2005), which means that baclofen has a very narrow pharmacological efficacy window. Baclofen has been in clinical use for the treatment of spasticity for over 30 years (Brogden et al., 1974). However, the muscle-relaxing properties of baclofen, together with its sedative and hypothermic effects, limit its widespread use as a tool in behavioural pharmacological studies (Dalvi and Rodgers, 1996). In the case of R-phenibut, we observed anti-nociceptive activity at a dose of 25 mg/kg, which is 5 times lower than the dose that induces muscle relaxation (Dambrova et al., 2008).

These data indicate that in neuropathic pain models, R-phenibut exhibits analgesic effects with a reduced risk of side effects compared with baclofen. In addition, the data clearly indicate that the analgesic activity of R-phenibut in neuropathic pain models is not mediated through $GABA_B$ receptor but rather through the α_2-δ_1 calcium channel subunit.

3.2.3. PTZ-induced seizure test

To test the anti-convulsant effects of these drugs, we used the PTZ-induced seizure test. Thirty minutes prior to i.v. injection of PTZ, saline, baclofen (2.5 mg/kg), R-phenibut (50 mg/kg), or GBP (50 mg/kg) was administered.

In the saline-treated mice, the doses of PTZ that-induced clonic and tonic seizures were 30±2 and 98±14 mg/kg, respectively. At a dose of 100 mg/kg, R-phenibut increased the

seizure-inducing dose of PTZ to 163%; however, this dose did not significantly differ from the control dose. At a dose of 50 mg/kg, R-phenibut did not have any effect on the seizure-inducing dose of PTZ. At doses of 50 and 100 mg/kg, GBP significantly increased the seizure-inducing dose of PTZ to 180% and 215%, respectively ($F_{(2,25)}$=6.608, p<0.01), compared to saline. At a dose of 2.5 mg/kg, baclofen did not influence the thresholds for PTZ-induced seizures (Table 2).

Table 2

Thresholds for PTZ-induced clonic and tonic seizures. R-phenibut, GBP and baclofen were administered i.p. at 30 min before i.v. injection of a 1% solution of PTZ.

	Saline	R-phenibut (mg/kg)		GBP (mg/kg)		Baclofen
		50	100	50	100	2.5 mg
Clonic seizures	30 ± 2	24 ± 3	27 ± 3	27 ± 3	27 ± 3	24 ± 4
Tonic seizures	98 ± 14	130 ± 14	157 ± 27	177 ± 20 *	216 ± 20 *	101 ± 20

The data were analysed by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. *p<0.05 vs. the saline group.

GBP has been demonstrated to be an efficacious antiepileptic drug in both animal models and clinical studies. It has been shown to exhibit anti-convulsant effects in numerous animal models employing rats and mice (Lothman et al., 1988; Loscher et al., 2000; Loscher et al., 1991). The anti-convulsant effects of GBP are associated with its binding to the α_2-δ_2 subunit of the VDCC (Barclay et al., 2001; Ivanov et al., 2004). In our study, we observed that R-phenibut only induced a trend towards anti-convulsant effects at high doses (100 mg/kg) (Table 2). These results indicate that either the μM affinity of R-phenibut for the α_2-δ_2 subunit of the VDCC is not sufficient to result in anti-convulsant effects or the pharmacological activity of R-phenibut is associated primarily with the α_2-δ_1 subunit, but not with the α_2-δ_2 subunit, of the VDCC. We also did not observe any anti-convulsant effects of a single administration of baclofen at a dose of 2.5 mg/kg in the PTZ-induced seizure test (Table 2). The effects of baclofen in seizure tests in rodents are controversial. Administration of R-baclofen has been shown to significantly decrease the severity of seizures and electrocortical epileptic discharges, as

induced by repeated sub-convulsant doses of PTZ but not by a single dose (De Serro et al., 2000). The anti-convulsant effect of baclofen has also been shown to depend on the experimental seizure model and on the age of the animals (Mares and Slamberová, 2006). Some studies have described pro-convulsant (Sokal and Large, 2001) and even convulsant (van Rijn et al., 1987) effects of baclofen. To summarize, the anti-convulsant activity of GBP is much more pronounced than that of the $GABA_B$ receptor agonists baclofen and R-phenibut.

Conclusions

In the present study, we characterized the binding of R-phenibut to the α_2-δ subunit of the VDCC, in addition to the anti-nociceptive and anti-convulsive effects of R-phenibut. Our results clearly showed that the binding affinity of R-phenibut for the α_2-δ subunit of the VDCC was 4 times higher than that for the $GABA_B$ receptor. Furthermore, R-phenibut reduced the nociceptive responses of mice during both phases of the formalin-induced paw-licking test. During the second phase, the $GABA_B$ receptor antagonist CGP35348 did not block the effects of R-phenibut. In addition, R- and S-phenibut alleviated the cold and thermal allodynia induced by CCI of the sciatic nerve.

In conclusion, R-phenibut is a clinically used $GABA_B$ receptor agonist that exhibits analgesic effects in the formalin-induced paw-licking test and on CCI-induced cold and thermal allodynia via the α_2-δ subunit of the VDCC without influencing PTZ-induced convulsions. Therefore, it may be an interesting and novel drug candidate to treat neuropathic pain.

Acknowledgements

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Fig. 1. Chemical structures of R-phenibut, S-phenibut, baclofen and GBP.

Fig. 2. The effects of R-phenibut (▲), S-phenibut (▼), baclofen (■) and GBP (●) on the binding of [^{3}H]GBP to the α_2-δ subunit of the VDCC in membranes prepared from male Wistar rat brains. The data represent at least three experiments performed in duplicate.

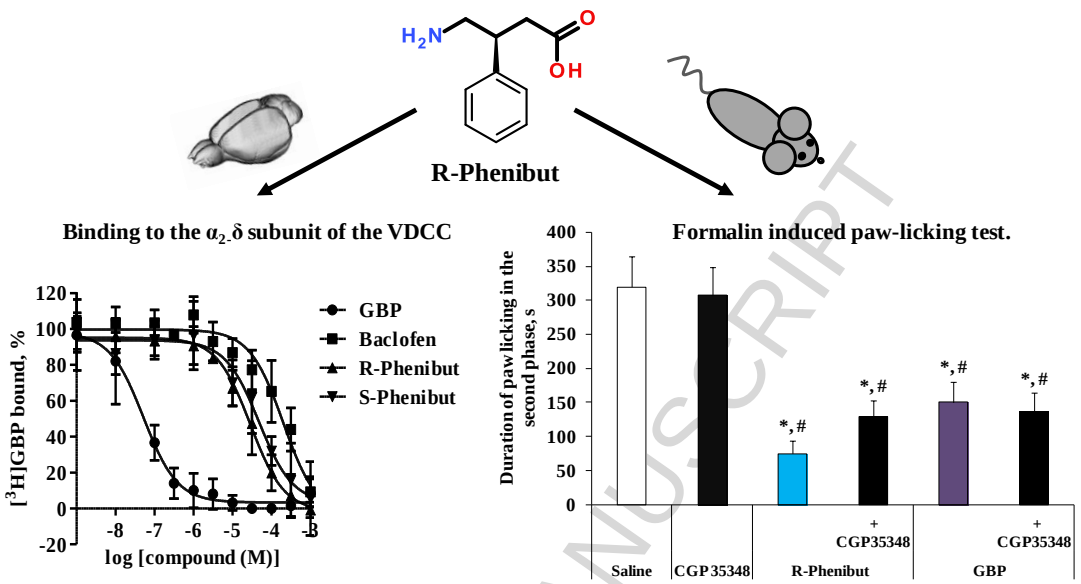
Fig. 3. Dose-dependent effects of R-phenibut on anti-nociceptive behaviours in the formalin-induced paw-licking test in mice. R-phenibut (10, 25 and 50 mg/kg) and GBP (50 mg/kg) were administered i.p. at 30 min before the test. Formalin (1.5%; 30 µL) was injected s.c. into the plantar surfaces of the 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. **(B)** The first phase was from 0 to 5 min and the second phase was from 15 to 40 min **(C)** after injection of formalin. The data are expressed as the mean±SEM (n=9–15) and were analysed by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. $^*p<0.05$ vs. the saline group.

Fig. 4. The effect of 100 mg/kg CGP35348 on the anti-nociceptive effects of R-phenibut and GBP in the formalin-induced paw-licking test in mice. R-phenibut (50 mg/kg) and GBP (50 mg/kg) were administered i.p. at 50 min before the test, and CGP35348 (100 mg/kg) was administered i.p. at 10 min before R-phenibut or GBP administration. Formalin (1.5%; 30 µL) was injected s.c. into the plantar surfaces of the right hind paws of the mice. Licking of the injected paw was recorded as the total time (s) spent licking within each of twelve 5-min bins for a total duration of 60 min. **(A)** The first phase was from 0 to 5 min, and **(B)** the second phase was from 15 to 40 min after injection of formalin. The data are expressed as the mean±SEM (n=6–12) and were analysed by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. $^*p<0.05$ vs. the saline group; $^\#p<0.05$ vs. the CGP35348 group.

Fig. 5. Effects of R- and S- phenibut on cold and mechanical allodynia in a CCI model in rats. Cold and mechanical allodynia were measured on the 6th day (baseline) after CCI and at 1 h and 4 h after drug administration on post-operative day 7. R-phenibut (25 and 50 mg/kg), S-phenibut (50 mg/kg) and GBP (100 mg/kg) were administered i.p. (**A**) Thermal allodynia was assessed in the CCI rats using the cold plate test. The data are

expressed as the latency in seconds. (**B**) Mechanical allodynia was assessed in the CCI rats using the electronic von Frey test. The data are expressed as the threshold in grams. (**C**) Areas under the curve (AUCs) were calculated from 1 h to 4 h after drug administration in the electronic von Frey test. The data are expressed as the mean±SEM (n=8) and were analysed by two-way and one-way ANOVA followed by Newman-Keuls multiple comparisons tests and Student's t-tests. *p<0.05 vs. the saline group.

Graphical abstract



Highlights

1. R-phenibut binds to the α_2-δ subunit of the voltage-dependent calcium channel (VDCC).

2. The binding affinity of R-phenibut for the α_2-δ subunit of the VDCC is 5 times higher than that for $GABA_B$ receptor.

3. R-phenibut exerts gabapentin-like anti-nociceptive effects in the formalin-induced paw-licking and CCI tests.

4. The anti-nociceptive effects of R-phenibut are not mediated through $GABA_B$ receptor.