Bioorganic & Medicinal Chemistry 18 (2010) 5938–5944

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and T-type calcium channel blocking activity of novel diphenylpiperazine compounds, and evaluation of in vivo analgesic activity

Yoo Lim Kam [a], Hee-Kyung Rhee [b], Hyewhon Rhim [c], Seung Keun Back [d], Heung Sik Na [d,*], Hea-Young Park Choo [a,*]

[a] School of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea
[b] Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Republic of Korea
[c] Life Sciences Division, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea
[d] Department of Physiology, Korea University College of Medicine, Seoul 136-705, Republic of Korea

ARTICLE INFO

Article history:
Received 3 May 2010
Revised 23 June 2010
Accepted 24 June 2010
Available online 30 June 2010

Keywords:
T-type calcium channel blocker
Diphenylpiperazine
Formalin test
Neuropathic pain

ABSTRACT

Novel diphenylpiperazine derivatives were synthesized and evaluated for their inhibitory activity against T-type calcium channel by whole-cell patch clamp recordings on HEK293 cells. Among the test compounds, **2** and **3d** were effective in decreasing the response to formalin in both the first and second phases and demonstrated antiallodynic effects in a rat model of neuropathic pain.

1. Introduction

Low-voltage-activated (LVA or T-type) calcium channels are expressed in peripheral and central neurons of the pain pathway. Three isoforms of T-type channel including $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}), and $Ca_v3.3$ (α_{1I}) have been identified and cloned. Recent evidence using the α_{1G}-deficient mice ($\alpha_{1G}^{-/-}$) suggested that $Ca_v3.1$ played a pivot role in the mediation of physiological and pathological pain.[1–5]

Mibefradil (Posicor®, Roche, Fig. 1), withdrawn from the market due to several drug interactions, is often described as a selective T-type calcium channel blocker (IC_{50} values: ~1 μM for $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$; ~10–20 μM for L-type) and used widely as a research tool to examine the functional role of T-type calcium channels. It was reported that mibefradil showed an antinociceptive property in both the first and second phases of the formalin response.[6–9] In the formalin test, biphasic behavioral responses are induced by formalin when injected subcutaneously. An acute nociceptive injury is thought to be involved in the first phase, while the second phase represents a model of central sensitization.[10]

Antipsychotic drugs such as flunarizine and pimozide are used clinically for the treatment of various psychiatric disorders (Fig. 1). Flunarizine is a neuroleptic of the diphenylpiperazine class. It has been described as one of the most potent neuronal T-type calcium channel blockers.[11] Flunarizine is known to block preferentially $Ca_v3.1$ and $Ca_v3.3$ channels (K_d = 0.53 and 0.84 μM, respectively), compared with $Ca_V3.2$ channels (K_d = 3.6 μM).[12] Pimozide, an antipsychotic, is a diphenylbutylpiperidine derivative that blocks dopaminergic D_2 receptors as other antipsychotics and neuroleptics. Pimozide markedly blocks the recombinant Ca_V3 channels (~40 nM) in the same concentration range as it binds to D_2 receptors (K_d ~29 nM).[12,13] Also, a mixed N-type and T-type calcium antagonist NP078585 (Fig. 1) was founded efficacious in animal models of chronic pain as well as attenuated ethanol intoxication.[14]

In attempts to have T-type channel blocking activity, we designed novel compounds possessing diphenylpiperazine moiety with a flexible alkyl chain as a linker attached with hydrophobic groups by mimicking flunarizine, pimozide, and mibefradil (Fig. 2). In this study, we report the synthesis of novel series of diphenylpiperazine derivatives and evaluation of their T-type calcium channel blocking activities in whole-cell patch clamp recordings. According to their T-type calcium channel blocking activities, two compounds (**2** and **3d**) were chosen for further in vivo studies of inflammatory pain and also evaluated their analgesic efficacies in a rat model of neuropathic pain.

* Corresponding authors.
E-mail addresses: hsna@korea.ac.kr (Heung Sik Na), hypark@ewha.ac.kr (Hea-Young Park Choo).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.06.082

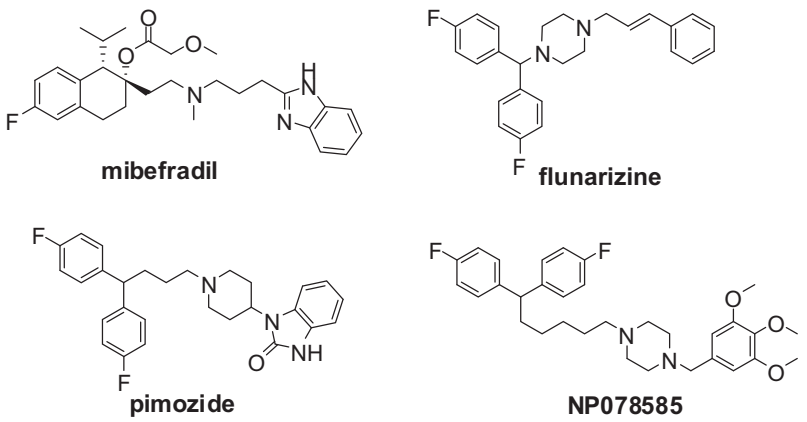


Figure 1. Structures of mibefradil, flunarizine, pimozide, and NP078585.

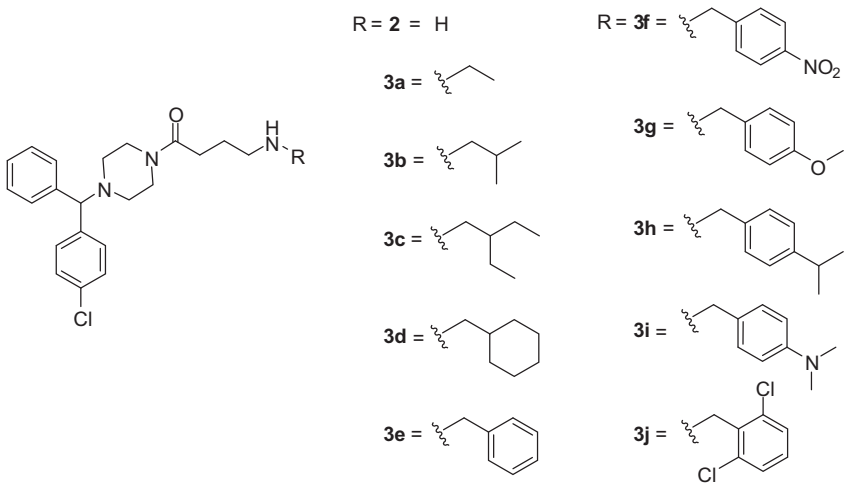
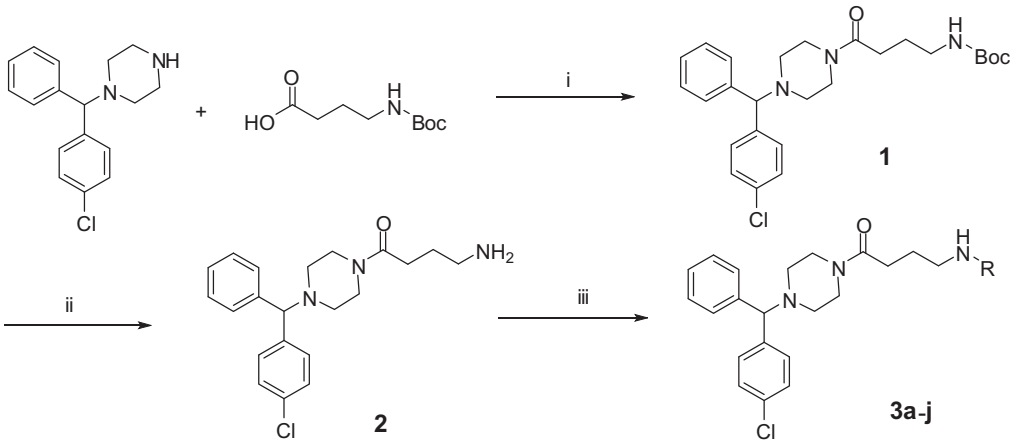


Figure 2. Structures of novel diphenylpiperazine derivatives.

2. Results and discussion

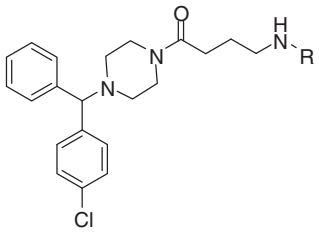
2.1. Chemistry

The synthetic procedures are consisted of two main reactions: coupling reaction and reductive amination as illustrated in Scheme 1. The coupling reaction of 1-((4-chlorobenzhydryl)piperazine with 4-(Boc-amino)butyric acid and PyBOP afforded compound **1**. *N*-Boc deprotection of compound **1** using HCl–dioxane gave compound **2**, which was treated with aldehydes and sodium triacetoxyborohydride to provide compounds **3a–j** via reductive amination. Each individual compound synthesized was character-



Scheme 1. Reagents and conditions: (i) PyBOP, DIEA, DMF, rt, 16 h; (ii) 4 M HCl–dioxane, $CHCl_3$, 3 h; (iii) aldehydes, $NaBH(OAc)_3$, TEA, DCE.

Table 1
Pharmacological data of diphenylpiperazine derivatives



Compds	R	HEK293 cell (T-type: α_{1G}) IC_{50}[a] (μM)	HEK293 cell (N-type: α_{1B}) % inhibition[b] (10 μM)
2	H	3.71 ± 0.67	10.03 ± 3.58
3a	Ethyl	0.42 ± 0.04	20.03 ± 5.68
3b	Isobutyl	0.36 ± 0.01	13.30 ± 4.50
3c	2-Ethylbutyl	1.54 ± 0.16	16.80 ± 5.30
3d	Cyclohexylmethyl	0.19 ± 0.01	84.86 ± 2.37
3e	Benzyl	1.67 ± 0.13	69.84 ± 1.85
3f	4-Nitrobenzyl	0.60 ± 0.08	88.75 ± 2.03
3g	4-Methoxybenzyl	16.10 ± 0.41	49.60 ± 9.17
3h	4-Isopropylbenzyl	0.28 ± 0.03	80.24 ± 2.68
3i	4-Dimethylaminobenzyl	2.32 ± 0.04	10.79 ± 1.38
3j	2,6-Dichlorobenzyl	1.02 ± 0.16	78.41 ± 4.04
	Mibefradil	1.34 ± 0.49	67.63 ± 1.21

[a] IC_{50} value was determined from the dose–response curve.
[b] % Inhibition value (±SE) was obtained by repeated procedures ($n \geqslant 3$) under patch–clamp assay.

ized by ^1H NMR, ^{13}C NMR and high resolution MS. The structures of different linkers and amines in both ends are illustrated in Table 1.

2.2. Effects on T-type and N-type calcium channels

The biological activity of the synthesized compounds was evaluated against HEK293 cells which stably express $Ca_v3.1$ (α_{1G}) T-type calcium channels. The IC_{50} value of Ca^{2+} current was measured. As a result, five novel compounds (**3a**, **3b**, **3d**, **3f**, and **3h**) inhibited T-type calcium current with IC_{50} values $\leqslant 1$ μM. Also the % inhibition of Ca^{2+} current on HEK293 cells which stably express $Ca_v3.1$ (α_{1B}) N-type calcium channels was measured at 10 μM concentration of the compounds, and three compounds (**3d**, **3f**, and **3h**) showed over 80% inhibition

Compounds **3a** and **3b** containing ethyl and isobutyl group, respectively, showed good selectivity on T-type over N-type calcium channel antagonism. On the other hand, **3d**, **3f**, and **3h**, with cyclohexyl, 4-nitrobenzyl or 4-isopropyl amines, respectively, inhibited not only T-type channel but also N-type calcium channel ranged from 80% to 89% at 10 μM. In this respect, T-type calcium channel antagonism is favored over N-type calcium channel inhibition when alkyl groups rather than benzyl groups are attached as R group. The compound **3d** with the most favorable T-type calcium channel blocking activity ($IC_{50} = 0.19 \pm 0.01$ μM) was further selected to verify in vivo analgesic effect. In addition, compound **2** with unsubstituted R group was also selected for in vivo study to compare analgesic effects between substituted and unsubstituted

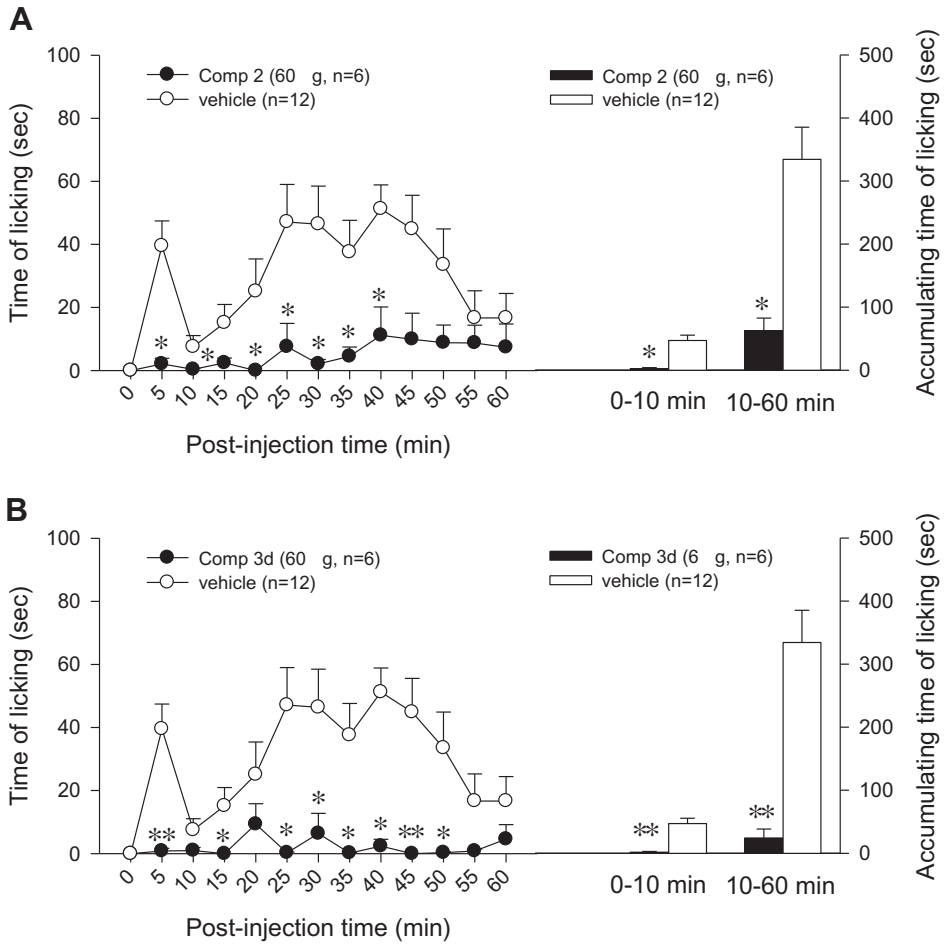


Figure 3. The effects of subcutaneous injection (60 μg) of compounds **2** (A) and **3d** (B) on formalin-induced pain response. Values represent the time (s) spent paw licking or biting in each 5-min interval following subcutaneous injection of formalin (5%, 50 μl). In the bar graph, values represent the response duration during the early (0–10 min) and late (10–60 min) phases after formalin injection. Data are expressed as means ± SEM. Significance of differences was analyzed by Student's unpaired *t*-test. *P <0.05.

R groups in rodent models of inflammatory and neuropathic pain. The observed pharmacological data are represented in Table 1.

2.3. Effects on formalin-induced pain

The rat formalin test, an inflammatory pain model, was examined for compounds **2** and **3d** (Fig. 3). The rats treated with the mixture of formalin and vehicle showed significant increase of the time spent in licking, shaking or biting the affected paw during both the early and late phases. However, compounds **2** and **3d** (60 μg, sc) injected with formalin clearly reduced the response duration in both phases. The response duration of pain behaviors in the vehicle, **2** and **3d** in the early phase was 47.05 ± 8.53, 2.41 ± 1.77, and 1.80 ± 1.14 s, respectively. In the late phase, the time spent in licking, shaking or biting the paw in each group was 334.21 ± 51.10, 62.57 ± 20.07, and 24.32 ± 14.08 s, respectively. This result indicates that compounds **2** and **3d** are effective in attenuating both nociceptive and inflammatory pain when treated locally.

2.4. Effects on neuropathic pain

Before nerve injury, animals rarely exhibited tail withdrawal responses to von Frey filaments, cold (4 °C) and warm (40 °C) water stimuli (Figs. 4 and 5). However, two weeks after nerve injury, rats exhibited significant decrease of tail withdrawal threshold and latencies in response to mechanical, cold and warm water stimuli, respectively [P <0.05 vs presurgical value (−1), one-way repeated measured ANOVA followed by Bonferroni's t-test]. We regarded these increased sensitivities as the signs of mechanical, cold and warm allodynia, and these neuropathic animals were subjected to the treatment of compound **2** or **3d**. As shown in Figure 4, Compound **2** (60 mg/kg, ip) and gabapentin (60 mg/kg, ip) significantly relieved the mechanical, cold and warm allodynia unlike vehicle group ($^{*}P$ <0.05 vs baseline, one-way repeated measured ANOVA followed by Bonferroni's t-test). On the other hand, compound **3d** (60 mg/kg, ip) significantly relieved the cold and warm, but not mechanical, allodynia (Fig. 5, $^{*}P$ <0.05 vs baseline, one-way repeated measured ANOVA followed by Bonferroni's t-test). Even though both **3d** and **2** showed analgesic effects, the behavioral results are not correlated with the biological activities of the compounds to T-(α_{1G}) type calcium channels. Compound **3d**, shown more robust inhibition against T-type calcium channels, less effectively relieved the neuropathic behaviors, especially mechanical allodynia than compound **2**. However, it is not unusual to observe this kind of results, because in vivo experiments the compounds were given ip and the results could be affected by pharmacokinetic factors of compounds, such as absorption, distribution, metabolism, and excretion. Additionally, compound **2** may possess other antiallodynic activities except the inhibitory effect against T-type or N-type calcium channels.

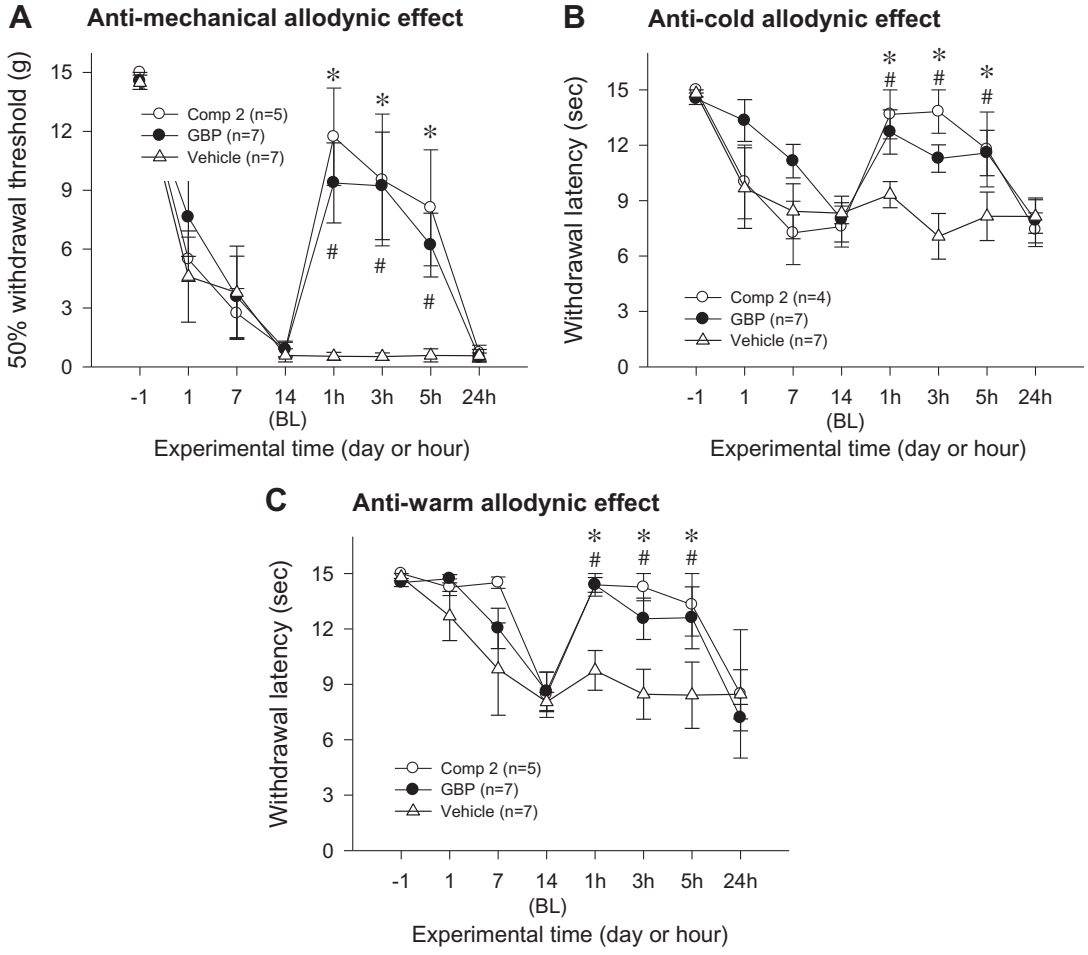


Figure 4. The effect of compound **2** (compd **2**) on mechanical (A), cold (B), and warm (C) allodynia induced by rat tail nerve injury. '−1' represents the 1 day prior to nerve injury. On 14 days after nerve injury, rats were given baseline (BL) test and then were subjected to the injection of compd **2** (60 mg/kg, ip), gabapentin (GBP, 60 mg/kg, ip) or vehicle (methyl pyrrolidone/Tween 80/saline = 1:1:8). Behavioral tests were re-performed 1, 3, 5, and 24 h after the injection. $^{*}P$ <0.05 (compd **2**); $^{\#}P$ <0.05 (GBP) versus BL (one-way repeated measured ANOVA followed by Bonferroni's t-test).

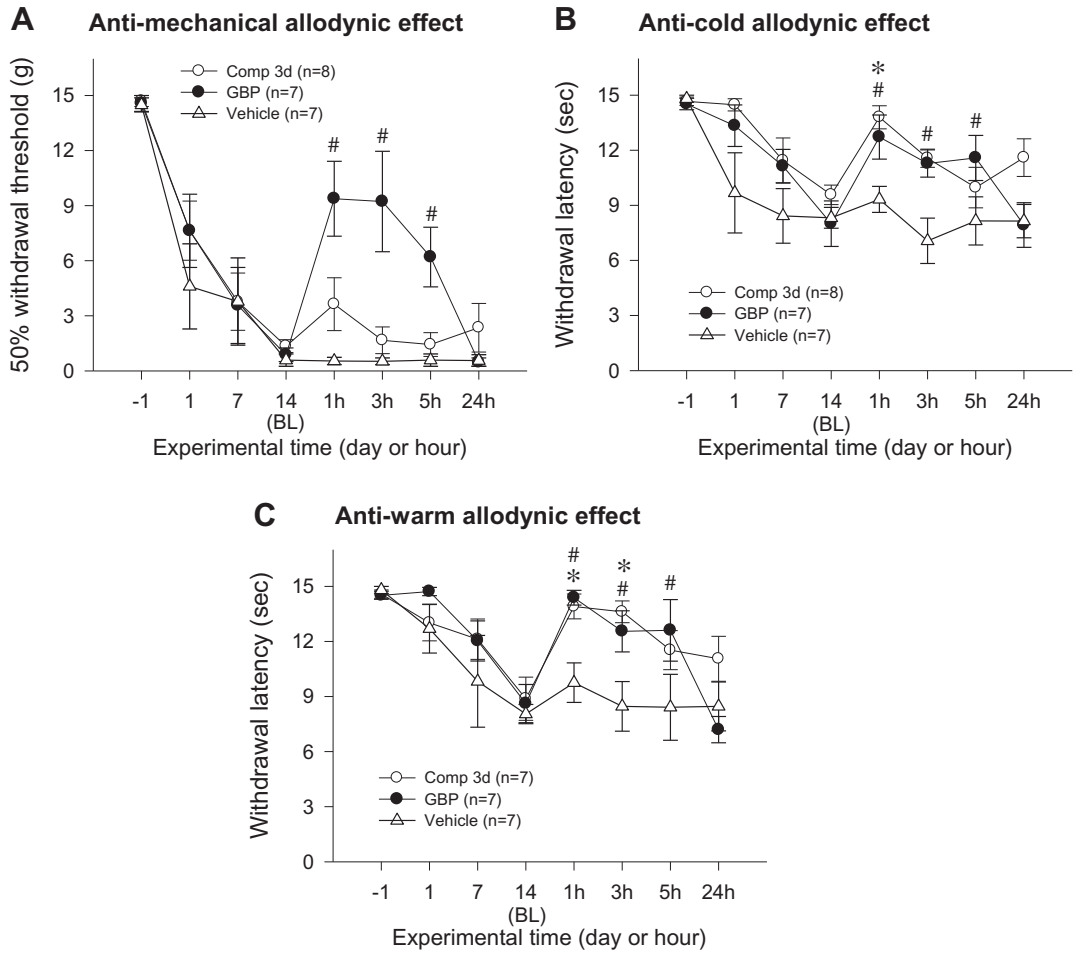


Figure 5. The effect of compound **3d** (compd **3d**) on mechanical (A), cold (B), and warm (C) allodynia induced by rat tail nerve injury. '−1' represents the 1 day prior to nerve injury. On 14 days after nerve injury, rats were given baseline (BL) test and then were subjected to the injection of compd **3d** (60 mg/kg, ip), gabapentin (GBP, 60 mg/kg, ip) or vehicle (methyl pyrrolidone/Tween 80/saline = 1:1:8). Behavioral tests were re-performed 1, 3, 5, and 24 h after the injection. $^{*}P$ <0.05 (compd **3d**); $^{\#}P$ <0.05 (GBP) versus BL (one-way repeated measured ANOVA followed by Bonferroni's *t*-test).

3. Conclusion

A series of novel diphenylpiperazine compounds were designed and synthesized by a simple synthetic method and evaluated their T-type calcium channel antagonism using path-clamp assay. Most of the test compounds inhibited T-type calcium current with IC_{50} values $\leqslant 1$ μM and compound **3d** showed the most favorable T-type calcium channel antagonism. In addition, compound **2** and **3d** produced statistically significant pain-relieving effects in both the first and second phases induced by formalin. Furthermore, compounds **2** and **3d** demonstrated analgesic effects in rodent model of neuropathic pain induced by peripheral nerve injury.

4. Experimental

4.1. Materials and methods

Melting points were measured on an electrothermal digital melting point (Buchi, Germany) without calibration. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian NMR AS and Varian Unity Inova 400 NMR spectrometers. Chemical shifts were reported in parts per million (δ) units relative to the solvent peak. The ^{1}H NMR data were reported as peak multiplicities: s for singlet; d for doublet; t for triplet; and m for multiplet. Coupling constants were recorded in hertz. Mass spectral data were obtained from the Korea Basic Science Institute (Daegu) on a Jeol JMS 700 high resolution mass spectrometer. Reagents were of commercial grade and were purchased from Sigma–Aldrich Co., Merck, Ducksan Pure Chemical Co.

4.2. Synthesis of *tert*-butyl 4-(4-((4-chlorophenyl)(phenyl) methyl)piperazin-1-yl)-4-oxobutylcarbamate (1)

In 3 mL of DMF, 4-(Boc-amino)butyric acid (2.46 mmol), 1-((4-chlorophenyl)(phenyl)methyl)piperazine (2.71 mmol) and PyBOP (2.71 mmol) were dissolved. Then, 4.92 mmol of DIEA was added and stirred at room temperature for 16 h. Twenty milliliters of 10% HCl was put into the reaction solution, and extracted with 30 mL of EtOAc. The organic layer was washed with 20 mL of 10% HCl, 20 mL of a saturated $NaHCO_3$ solution twice and with 20 mL of a saturated NaCl solution twice. The organic layer was collected, dried over anhydrous $MgSO_4$, and filtered. The organic solvent in the filtrate was removed under reduced pressure. The residue was recrystallized from EtOAc to afford desired compound. White solid, yield: 80%; ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.46–7.53 (m, 4H), 7.29–7.35 (m, 4H), 7.19–7.22 (m, 1H), 5.96 (br s, 1H), 4.39 (s, 1H), 3.53–3.56 (m, 4H), 3.07 (q, J = 6.4 Hz, 2H), 2.32–2.40 (m, 6H), 1.70–1.77 (m, 2H), 1.38 (s, 9H); HR-FABMS calcd for $C_{26}H_{35}O_3N_3Cl$ $(M+H)^+$: 472.2367, found: 472.2365.

4.3. Synthesis of 4-amino-1-(4-((4-chlorophenyl)(phenyl) methyl)piperazin-1-yl)butan-1-one (2)

$CHCl_3$ (5 mL) and 4 M HCl–dioxane (5 mL) were added to compound **1** (0.76 mmol) and was allowed to stand for 3 h. The solvent and excess acid were removed under reduced pressure. White solid, yield: 99%; mp 115–118 °C; ^{1}H NMR (D_2O, 400 MHz) δ 7.63–7.66 (m, 4H), 7.51–7.57 (m, 5H), 5.41 (s, 1H), 3.82–3.87 (m, 4H), 3.28–3.33 (m, 4H), 3.04 (t, J = 7.6 Hz, 2H), 2.60 (t, J = 7.2 Hz, 2H), 1.92–1.99 (m, 2H); ^{13}C NMR (D_2O, 100 MHz) δ 172.96, 135.35, 133.63, 132.50, 130.10, 129.98, 129.75, 128.14; HR-FABMS calcd for $C_{21}H_{27}ON_3Cl$ $(M+H)^+$: 372.1843, found: 372.1842.

4.4. General procedure for diphenylpiperazine derivatives (3a–j)

In 1,2-dichloroethane (DCE) 9 mL, 4-amino-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)butan-1-one (**2**, 0.5 mmol) and aldehydes (0.5 mmol) were dissolved. Then, 0.7 mmol of sodium triacetoxyborohydride ($NaBH(OAc)_3$) and 0.5 mmol of triethylamine were added and reacted at room temperature overnight under nitrogen. The reaction mixture was quenched by saturated $NaHCO_3$ solution and extracted with 30 mL of EtOAc. The organic layer was collected, dried over anhydrous $MgSO_4$, and filtered. The organic solvent in the filtrate was removed under reduced pressure.

4.4.1. 1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-4-(ethylamino)butan-1-one (3a)

Brown solid, yield: 79%; mp 141–143 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34–7.38 (m, 4H), 7.21–7.30 (m, 5H), 4.21 (s, 1H), 3.58–3.63 (m, 2H), 3.44–3.48 (m, 2H), 2.64–2.67 (m, 2H), 2.44–2.56 (m, 2H), 2.31–2.38 (m, 6H), 1.74–1.82 (m, 2H), 1.05 (t, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.19, 141.56, 140.72, 131.47, 129.14, 128.82, 128.76, 127.79, 127.44, 75.10, 51.89, 51.47, 45.42, 41.85, 29.22, 26.29, 14.21; HR-FABMS calcd for $C_{23}H_{31}ON_3Cl$ $(M+H)^+$: 400.2156, found: 400.2156.

4.4.2. 1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-4-(isobutylamino)butan-1-one (3b)

Pale yellow solid; yield: 43%; mp 66–68 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34–7.38 (m, 4H), 7.21–7.31 (m, 5H), 4.21 (s, 1H), 3.58–3.64 (m, 2H), 3.44–3.48 (m, 2H), 2.64 (t, J = 6.8 Hz, 2H), 2.32–2.42 (m, 6H), 1.71–1.84 (m, 4H), 1.26 (t, J = 7.2 Hz, 1H), 0.90 (d, J = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.25, 141.63, 140.86, 132.91, 129.23, 128.91, 128.85, 127.88, 127.52, 75.29, 57.08, 52.04, 51.61, 48.98, 45.78, 41.85, 31.02, 27.65, 20.68; HR-FABMS calcd for $C_{25}H_{35}ON_3Cl$ $(M+H)^+$: 428.2469, found: 428.2469.

4.4.3. 4-(2-Ethylbutylamino)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)butan-1-one (3c)

Green solid; yield: 25%; mp 91–93 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34–7.4 (m, 4H), 7.21–7.34 (m, 5H), 4.21 (s, 1H), 3.39–3.66 (m, 6H), 2.31–2.38 (m, 6H), 1.88–2.0 (m, 2H), 1.80–1.82 (m, 1H), 1.60–1.69 (m, 4H), 1.41–1.47 (m, 2H), 0.78–0.96 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.29, 141.54, 140.80, 132.77, 129.13, 128.78, 128.72, 128.51, 127.77, 127.39, 75.18, 52.43, 51.97 51.53, 49.44, 45.68, 41.70, 40.43, 30.93, 25.06, 23.89, 10.86; HR-FABMS calcd for $C_{27}H_{39}ON_3Cl$ $(M+H)^+$: 456.2782, found: 456.2778.

4.4.4. 1-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-4-(cyclohexylmethylamino)butan-1-one (3d)

White solid; yield: 20%; mp 136–139 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34–7.38 (m, 4H), 7.21–7.31 (m, 5H), 4.21 (s, 1H), 3.58–3.62 (m, 2H), 3.44–3.48 (m, 2H), 2.77 (t, J = 6.8 Hz, 2H), 2.52 (d, J = 6.4 Hz, 2H), 2.42 (t, J = 7.0 Hz, 2H), 2.35–2.39 (m, 4H), 1.78–1.85 (m, 2H), 1.62–1.74 (m, 4H), 1.12–1.28 (m, 4H), 0.84–0.96 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.59, 141.59, 140.79, 133.15, 129.32, 129.07, 129.01, 127.98, 127.71, 75.25, 54.27, 51.83, 51.48, 48.60, 35.02, 31.91, 31.14, 30.91, 26.17, 25.53; HR-FABMS calcd for $C_{28}H_{37}ON_3Cl$ $(M+H)^+$: 468.2782, found: 468.2778.

4.4.5. 4-(Benzylamino)-1-(4-((4-chlorophenyl)(phenyl) methyl)piperazin-1-yl)-butan-1-one (3e)

Yellow oil; yield: 84%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.21–7.42 (m, 14H), 4.21 (s, 1H), 3.9 (s, 2H), 3.56–3.62 (m, 2H), 3.42–3.46 (m, 2H), 2.80 (t, J = 6.4 Hz, 2H), 2.41 (t, J = 6.8 Hz, 2H), 2.32–2.38 (m, 4H), 1.88–1.94 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.13, 141.57, 140.81, 139.52, 130.60, 129.13, 128.78, 128.72, 128.59, 128.41, 128.28, 128.04, 127.78, 127.38, 127.10, 75.17, 51.98, 51.51, 48.49, 45.63, 41.66, 30.61, 26.55; HR-FABMS calcd for $C_{28}H_{33}ON_3Cl$ $(M+H)^+$: 462.2312, found: 462.2316.

4.4.6. 4-(4-Nitrobenzylamino)-1-(4-((4-chlorophenyl)(phenyl) methyl)piperazin-1-yl)butan-1-one (3f)

Yellow oil; yield: 76%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.24 (d, J = 8.8 Hz, 1H), 8.16 (d, J = 8.8 Hz, 1H), 7.55 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.21–7.37 (m, 9H), 4.21 (s, 1H), 3.88 (s, 2H), 3.60–3.65 (m, 2H), 3.44–3.48 (m, 2H), 2.66 (t, J = 6.8 Hz, 2H) 2.32–2.38 (m, 6H), 1.81–1.88 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.00, 149.02, 141.73, 141.60, 140.85, 132.85, 129.19, 128.86, 128.82, 127.84, 127.49, 124.34, 123.91, 75.26, 52.06, 51.62, 48.75, 45.72, 41.78, 30.63, 26.41; HR-FABMS calcd for $C_{28}H_{32}O_3N_4Cl$ $(M+H)^+$: 507.2163, found: 507.2162.

4.4.7. 4-(4-Methoxybenzylamino)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)butan-1-one (3g)

Pale yellow gel; yield: 19%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.19–7.38 (m, 11H), 6.84–6.91 (m, 2H), 4.21 (s, 1H), 3.90 (s, 2H), 3.81 (s, 3H), 3.55–3.60 (m, 2H), 3.43–3.49 (m, 2H), 2.80–2.88 (m, 2H), 2.30–2.47 (m, 6H), 1.92–2.02 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.37, 160.85, 141.66, 140.90, 132.85, 312.39, 132.06, 129.68, 129.40, 129.21, 128.87, 128.82, 127.86, 127.47, 114.40, 113.86, 75.27, 55.65, 52.07, 51.62, 48.68, 45.74, 41.76, 30.96, 25.54; HR-FABMS calcd for $C_{29}H_{35}O_2N_3Cl$ $(M+H)^+$: 492.2418, found: 492.2420.

4.4.8. 4-(4-Isopropylbenzylamino)-1-(4-((4-chlorophenyl) (phenyl)methyl)piperazin-1-yl)butan-1-one (3h)

Pale yellow solid; yield: 81%; mp 65–66 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.61 (d, J = 8.0 Hz, 1H), 7.16–7.40 (m, 11H), 7.18 (d, J = 8.0 Hz, 1H) 4.21 (d, J = 6.4 Hz, 1H), 3.74 (s, 2H), 3.58–3.64 (m, 2H), 3.44–3.48 (m, 2H), 2.86–2.96 (m, 1H), 2.68 (t, J = 6.8 Hz, 2H), 2.32–2.41 (m, 6H), 1.98–2.04 (m, 2H), 1.23–1.30 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.40, 147.72, 141.69, 140.91, 132.91, 130.11, 129.24, 128.91, 128.84, 128.27, 127.89, 127.51, 127.25, 126.81, 126.55, 126.25, 75.33, 53.60, 52.12, 51.67, 48.80, 45.77, 41.79, 33.88, 31.00, 25.53, 24.16; HR-FABMS calcd for $C_{31}H_{39}ON_3Cl$ $(M+H)^+$: 504.2782, found: 504.2776.

4.4.9. 4-(4-(Dimethylamino)benzylamino)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)butan-1-one (3i)

Light brown solid; yield: 33%; mp 85–87 °C; ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.45–7.72 (m, 5H), 7.16–7.35 (m, 5H), 6.70–6.82 (m, 3H), 4.31 (s, 1H), 3.50–3.58 (m, 6H), 3.01 (s, 6H), 2.88–2.9 (m, 2H), 2.38 (t, J = 7.4 Hz, 2H), 2.31–2.36 (m, 4H), 1.86–1.93 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.59, 150.54, 141.82, 141.03, 132.96, 129.65, 129.52, 129.32, 129.17, 128.95, 128.81, 127.97, 127.56, 124.51, 112.80, 111.80, 75.43, 52.23, 51.80, 45.89, 41.83, 40.84, 40.27, 30.88, 27.00; HR-FABMS calcd for $C_{30}H_{38}ON_4Cl$ $(M+H)^+$: 505.2734, found: 505.2738.

4.4.10. 4-(2,6-Dichlorobenzylamino)-1-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)butan-1-one (3j)

Pale yellow gel; yield: 25%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.19–7.38 (m, 12H), 4.21 (s, 1H), 3.74–3.78 (m, 2H), 3.58–3.64 (m, 2H), 3.44–3.49 (m, 2H), 2.44 (t, J = 7.6 Hz, 2H), 2.32–2.39 (m, 4H), 2.04–2.12 (m, 2H), 1.79–1.84 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.86, 141.69, 140.90, 136.48, 136.16, 135.93, 132.97, 129.92, 129.31, 128.97, 128.92, 128.68, 128.62, 127.93, 127.58, 75.19, 51.94, 51.55, 45.78, 31.03, 29.85; HR-FABMS calcd for $C_{28}H_{31}ON_3Cl_3$ $(M+H)^+$: 530.1533, found: 530.1530.

4.5. Effect on T-type calcium channel

For the recordings of α_{1G} T-type Ca^{2+} currents and N-type Ca^{2+} channels, the standard whole-cell patch–clamp method was utilized.[15] Briefly, borosilicate glass electrodes with a resistance of 3–4 MΩ were pulled and filled with the internal solution containing (in mM): 130 KCl, 11 EGTA, 5 Mg-ATP, and 10 Hepes (pH 7.4). The external solution contained (in mM): 140 NaCl, 2 $CaCl_2$, 10 Hepes, and 10 glucose (pH 7.4). α_{1G} T-type Ca^{2+} currents were evoked every 15 s by a 50 ms depolarizing voltage step from −100 mV to −30 mV. The molar concentrations of test compounds required to produce 50% inhibition of peak currents (IC_{50}) were determined from fitting raw data into dose–response curves. The current recordings were obtained using an EPC-9 amplifier and Pulse/Pulsefit software program (HEKA, Germany).

4.6. Nociceptive and inflammatory pain

4.6.1. Behavioral testing for formalin test

To investigate the effect of compounds **2** and **3d** on both nociceptive and inflammatory pain, the formalin test was carried out. Each rat was placed in the observation chamber (20 × 20 × 20 cm) equipped with a mirror placed behind the chamber to allow an unobstructed view of the paw for a 30 min habituation period. Thereafter, animals were assigned to the two groups; the first group was subjected to the subcutaneous injection of the mixture of formalin (5%, 50 μl) and vehicle (Methyl pyrrolidone: Tween80:-Saline = 1:1:8, 10 μl) under the plantar surface of the right hind paw using a 30-gage syringe, the second group was treated with the mixture of formalin (5%, 50 μl) plus test compounds (60 μg/10 μl) by the same way described above. After the injection, animals were returned to the chamber immediately and the time spent in licking, shaking or biting the injected paw was recorded for every 5 min over a 60-min period. The early phase was defined as the first 10 min after formalin injection, and the late phase was the following 50 min.[10,16] The observations in this test were carried out by three investigators who were unaware of the treatment status.

4.7. Neuropathic pain

4.7.1. Tail nerve injury

To examine the effect of compounds **2** and **3d** on neuropathic pain, tail nerve injury (TNI) model was used. Tail nerve injury was based on the procedure previously described by Na and coworkers.[17,18] Briefly, under enflurane anesthesia, animals were subjected to unilateral transection of the superior and inferior caudal trunks at the level between the S1 and the S2 spinal nerves. To prevent possible rejoining of the proximal and distal ends of the severed trunks, pieces of nerve about 2 mm in length were removed from the distal nerve ends. This surgery injured the S1 spinal nerve which innervates the tail.

4.7.2. Behavioral testing for neuropathic pain

Mechanical allodynia of rat tails was assessed by measuring the withdrawal thresholds in response to a series of calibrated von Frey filaments (3.92, 5.88, 9.80, 19.60, 39.20, 58.80, 78.40, and 147.00 mN, Stoeling, Wood Dale, IL, USA; equivalent to 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g). The 50% withdrawal threshold was determined using the up-down method.[19] In brief, testing was initiated with a filament whose bending force was 19.60 mN in the middle of the series. When a withdrawal response was obtained, next weaker filament was used, whereas next stronger filament was administered when no response was obtained. Interpolation of the 50% threshold was carried out using the Dixon method.[20] A brisk tail withdrawal to von Frey filament application was regarded as a positive response. Testing for cold or warm allodynia was performed by measuring tail withdrawal latency to cold (4 °C) or warm (40 °C) water stimulation, respectively.[18] After immersing the tail into cold or warm water bath, the tail was continuously observed to find out whether it moved abruptly, and the latency of tail movement was measured within a cut-off time of 15 s. An abrupt tail movement within the cut-off time was considered as a positive withdrawal response, whereas a lack of tail movement until the cut-off time or slow tail movement within the cut-off time was considered to be not a positive response. The testing was repeated five times with 5 min intervals, and the average latency of tail response was calculated. At 2 weeks after TNI, rats were given a baseline test and thereafter test compounds (60 mg/kg), gabapentin (60 mg/kg), which is widely used for the treatment of neuropathic pain or vehicle (Methyl pyrrolidone:Tween80:Saline = 1:1:8) was administered intraperitoneally. Behavioral tests for mechanical, cold and warm allodynia were conducted at 1, 3, and 5 h after the injection. Behavioral testing was carried out by an investigator who was unaware of the treatment status of the rats.

4.8. Statistical analysis

All data are presented as mean ± SEM. Student's unpaired t-test, repeated one-way ANOVA (Bonferroni t-test) and paired t-test were used wherever appropriate. A difference of P <0.05 was considered to be statistically significant.

Acknowledgments

This study was supported in part by the Korea Research Foundation (Grant KRF-2004-E00406) and Grant (M103KV010015-08K2201-01510) from Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, the Republic of Korea.

References and notes

1. Catterall, W. A. *Annu. Rev. Cell Dev. Biol.* **2000**, *16*, 521.
2. Perez-Reyes, E. *Physiol. Rev.* **2003**, *83*, 117.
3. Shin, H.-S.; Cheong, E.-J.; Choi, S.; Lee, J.; Na, H. S. *Curr. Opin. Pharmacol.* **2008**, *8*, 33.
4. Kim, D.; Park, D.; Choi, S.; Lee, S.; Sun, M.; Kim, C.; Shin, H.-S. *Science* **2003**, *302*, 117.
5. Snutch, T. P.; David, L. S. *Drug Dev. Res.* **2006**, *67*, 404.
6. Barton, M. E.; Eberle, E. L.; Shannon, H. E. *Eur. J. Pharmacol.* **2005**, *521*, 79.
7. McGiven, J. G. *Drug Discovery Today* **2006**, *11*, 245.
8. Massie, B. M. *Am. J. Cardiol.* **1997**, *80*, 231.
9. Mullins, M. E.; Horowitz, B. Z.; Linden, D. H.; Smith, G. W.; Norton, R. L.; Stump, J. *JAMA* **1998**, *280*, 157.
10. Dubuisson, D.; Dennis, S. G. *Pain* **1977**, *4*, 161.
11. Akaike, N. *Comp. Biochem. Physiol. C* **1991**, *98*, 31.
12. Santi, C. M.; Cayabyab, F. S.; Sutton, K. G.; McRory, J. E.; Mezeyova, J.; Hamming, K. S.; Parker, D.; Stea, A.; Snutch, T. P. *J. Neurosci.* **2002**, *22*, 396.
13. Richelson, E.; Souder, T. *Life Sci.* **2000**, *68*, 29.
14. Newton, P. M.; Zeng, L.; Wang, V.; Connolly, J.; Wallace, M. J.; Kim, C.; Shin, H.-S.; Belardetti, F.; Snutch, T. P.; Messing, R. O. *J. Neurosci.* **2008**, *28*, 11712.
15. Kim, T.; Choi, J.; Kim, S.; Kwon, O.; Nah, S. Y.; Han, Y. S.; Rhim, H. *Biochem. Biophys. Res. Commun.* **2004**, *324*, 401.
16. Haley, J.; Ketchum, S.; Dickenson, A. *Eur. J. Pharmacol.* **1990**, *191*, 437.
17. Back, S. K.; Kim, J. S.; Hong, S. K.; Na, H. S. *Neuroreport* **2003**, *14*, 1623.
18. Na, H. S.; Han, J. S.; Ko, K. H.; Hong, S. K. *Neurosci. Lett.* **1994**, *177*, 50.
19. Chaplan, S. R.; Bach, F. W.; Pogrel, J. W.; Chung, J. M.; Yaksh, T. L. *J. Neurosci. Methods* **1994**, *53*, 55.
20. Dixon, W. J. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 441.