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# Antinociceptive Effects of Central Administration of the Endogenous Cannabinoid Receptor Type 1 Agonist VDPVNFKLLSH-OH [(m)VD-hemopressin($\alpha$)], an N-Terminally Extended Hemopressin Peptide

Zheng-lan Han,[1] Quan Fang,[1] Zi-long Wang, Xu-hui Li, Ning Li, Xue-mei Chang, Jia-xin Pan, Hong-zhu Tang, and Rui Wang

*Key Laboratory of Preclinical Study for New Drugs of Gansu Province, and Institute of Physiology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, People's Republic of China*

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## ABSTRACT

The cannabinoid system has been demonstrated to modulate the acute and chronic pain of multiple origins. Mouse VD-hemopressin ($\alpha$) [(m)VD-Hp$\alpha$], an 11-residue $\alpha$-hemoglobin–derived peptide, was recently reported to function as a selective agonist of the cannabinoid receptor type 1 ($CB_1$) in vitro. To characterize its behavioral and physiological properties, we investigated the in vivo effects of (m)VD-Hp$\alpha$ in mice. In the mouse tail-flick test, (m)VD-Hp$\alpha$ dose-dependently induced antinociception after supraspinal ($EC_{50}$ = 6.69 nmol) and spinal ($EC_{50}$ = 2.88 nmol) administration. The antinociceptive effects of (m)VD-Hp$\alpha$ (intracerebroventricularly and intrathecally) were completely blocked by *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3- carboxamide (AM251; $CB_1$ antagonist), but not by 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl (4-methoxyphenyl)-methanone (AM630; $CB_2$ antagonist) or naloxone (opioid antagonist), showing its selectivity to the $CB_1$ receptor. Furthermore, the central nervous system (CNS) effects of (m)VD-Hp$\alpha$ were evaluated in body temperature, locomotor activity, tolerance development, reward, and food intake assays. At the highly antinociceptive dose (3 × $EC_{50}$), (m)VD-Hp$\alpha$ markedly exerted hypothermia and hypoactivity after supraspinal administration. Repeated intracerebroventricular injection of (m)VD-Hp$\alpha$ resulted in both development of tolerance to antinociception and conditioned place aversion. In addition, central injection of (m)VD-Hp$\alpha$ dose-dependently stimulated food consumption. These findings demonstrate that this novel cannabinoid peptide agonist induces $CB_1$-mediated central antinociception with some CNS effects, which further supports a $CB_1$ agonist character of (m)VD-Hp$\alpha$. Moreover, the current study will be helpful to understand the in vivo properties of the endogenous peptide agonist of the cannabinoid $CB_1$ receptor.

## Introduction

Pain is one of the most widespread and difficult syndromes of humans. Cannabinoid receptors are the attractive therapeutic targets for pain management (Richardson, 2000; Pacher et al., 2006). To date, two types of cannabinoid receptors, cannabinoid receptor type 1 ($CB_1$) and type 2 ($CB_2$), have been identified (Pertwee, 1997; Pacher et al., 2006). The $CB_1$ receptor is expressed in neurons throughout the central and peripheral nervous system, especially in the areas that are involved in pain modulation, including the periaqueductal gray and the dorsal horn of the spinal cord (Richardson, 2000; Agarwal et al., 2007; Guindon and Beaulieu, 2009). In contrast, the $CB_2$ receptor is predominantly present in immune cells. However, recent studies demonstrated that activation of the $CB_2$ receptor also produced acute antinociception (Richardson, 2000; Ibrahim et al., 2006; Pacher et al., 2006; Guindon and Beaulieu, 2009).

It is well known that the cannabinoid receptors are activated by synthetic agonists [e.g., $\Delta^8$-tetrahydrocannabinol dimethyl heptyl (Hu-210)], phytocannabinoids (e.g., $\Delta^9$-tetrahydrocannabinol), and lipid endocannabinoids (e.g., 2-arachidonoylglycerol) derived from membrane phospholipids (Pacher et al., 2006; Blankman and Cravatt, 2013). The endocannabinoid system is traditionally

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[1]Z.-I.H. and Q.F. contributed equally to this work.
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**ABBREVIATIONS:** AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3- carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl(4-methoxyphenyl)-methanone; ANOVA, analysis of variance; AUC, area under the curve; $CB_1$, cannabinoid receptor type 1; $CB_2$, cannabinoid receptor type 2; CNS, central nervous system; CP55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; CPP, conditioned place preference; Fmoc, *N*-fluorenylmethoxycarbonyl; Hu-210, $\Delta^8$-tetrahydrocannabinol dimethyl heptyl; MPE, percent maximum possible effect; (m)VD-Hp$\alpha$, mouse VD-hemopressin($\alpha$); (m)RVD-Hp$\alpha$, mouse RVD-hemopressin($\alpha$); SAB378, naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone; SR141716A, *N*-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; WIN55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone.

thought to be modulated by the lipophilic endocannabinoids. However, recent findings indicated that endocannabinoid receptors were also regulated by hemopressin peptides (Heimann et al., 2007; Gomes et al., 2009; Bomar and Galande, 2013). Hemopressin is a nonapeptide derived from the $\alpha_1$-chain of rat hemoglobin. This nonapeptide was reported to function as an endogenous inverse agonist or antagonist of the $CB_1$ receptor (Heimann et al., 2007; Dodd et al., 2010). Subsequent findings revealed that three related peptides of hemopressin, mouse RVD-hemopressin($\alpha$) [(m)RVD-Hp$\alpha$], mouse VD-hemopressin($\alpha$) [(m)VD-Hp$\alpha$], and mouse VD-hemopressin ($\beta$), which were identified in mouse brain extracts, behaved as agonists of cannabinoid receptors with different selectivities toward $CB_1$ and $CB_2$ receptors in vitro (Gomes et al., 2009). In the $G_{\alpha i16}$-facilitated $Ca^{2+}$ release assay, both (m)RVD-Hp$\alpha$ and (m)VD-Hp$\alpha$ acted as the $CB_1$ agonists, whereas mouse VD-hemopressin($\beta$) functioned as a cannabinoid agonist at both $CB_1$ and $CB_2$ receptors on human embryonic kidney 293 cells coexpressing a chimeric $G_{16}/G_{i3}$ with either the $CB_1$ or $CB_2$ cannabinoid receptor (Gomes et al., 2009). However, compared with (m)RVD-Hp$\alpha$, (m)VD-Hp$\alpha$ caused a lesser increase in intracellular $Ca^{2+}$ level in cells coexpressing a chimeric $G_{16}/G_{i3}$ with the $CB_2$ receptor, implying that (m)VD-Hp$\alpha$ is a highly selective $CB_1$ agonist (Gomes et al., 2009). Furthermore, the data obtained from studies of the phosphorylation level of extracellular signal-regulated kinase 1/2 or release of intracellular $Ca^{2+}$ demonstrated that the signal transduction pathway activated by these peptides was distinct from that of 2-arachidonoylglycerol and Hu-210 (Gomes et al., 2009).

A number of biological studies suggested that cannabinoids were involved in pain modulation, suppression of locomotor activity, hypothermia, catalepsy, food intake, and cardiovascular actions (Pacher et al., 2006; Bushlin et al., 2010; Blankman and Cravatt, 2013). The pharmacological and behavioral profiles of several nonpeptidic agonists of cannabinoid receptors have been well investigated (Pacher et al., 2006). Although the $CB_1$ inverse agonist or antagonist hemopressin was reported to elicit inhibition of food intake, hypotensive and antinociceptive effects (Rioli et al., 2003; Blais et al., 2005; Dale et al., 2005; Heimann et al., 2007; Dodd et al., 2010), the behavioral and physiological effects of the endogenous peptide agonists of the cannabinoid system, have not been characterized. Thus, further study of hemopressin and related peptides could be helpful to characterize the physiological role of the endocannabinoid system.

The $CB_1$ receptor is predominantly located in the mammalian brain (Mechoulam and Parker, 2013). A number of studies reported that the cannabinoid system plays important roles in the modulation of acute nociceptive stimulation and in chronic pain processes (Richardson, 2000; Pertwee, 2001; Guindon and Beaulieu, 2009; Bushlin et al., 2010). In addition, administration of hemopressin caused significant nonopioid antinociception (Dale et al., 2005; Heimann et al., 2007). Therefore, in the present study, acute antinociceptive profiles and related central nervous system (CNS) effects of the endogenous agonist (m)VD-Hp$\alpha$, an N-terminally extended hemopressin with high selectivity toward the $CB_1$ receptor, were investigated in mice.

## Materials and Methods

**Animals.** The experiments were performed on male Kunming strain mice from the Experimental Animal Center of Lanzhou University (Lanzhou, China). The mice were housed in a temperature-controlled room (22 ± 1°C). Food and water were freely available until the onset of the behavioral test. All animals were cared for and experiments were carried out in accordance with the European Community guidelines for the use of experimental animals (86/609/EEC). All of the protocols in this study were approved by the Ethics Committee of Lanzhou University.

**Chemicals.** In the present study, (m)VD-Hp$\alpha$ (VDPVNFKLLSH-OH) was prepared by manual solid-phase synthesis using standard *N*-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-protected amino acids [GL Biochem (Shanghai) Ltd., Shanghai, China] were coupled to a Fmoc-His(Trt)-Wang resin (Tianjin Nankai Hecheng Science & Technology Co., Ltd., Tianjin, China). Gel filtration (Sephadex G-25; Amersham Pharmacia Biotech (China)Ltd., Shanghai, China) was performed to desalt the crude peptides. The desalted peptide was purified by preparative reversed-phase high-performance liquid chromatography using a Waters Delta 600 system (Milford, MA) coupled to a UV detector. Fractions containing the purified peptides were pooled and lyophilized. The purity of the peptide was established by analytical high-performance liquid chromatography. The molecular weight of the peptide was confirmed by an electrospray ionization mass spectrometer (ESI-Q-TOF maXis-4G; Bruker Daltonics, Germany).

In addition, the cannabinoid agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN55,212-2) and naloxone were obtained from Sigma-Aldrich (St. Louis, MO). The selective cannabinoid receptor antagonists *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3- carboxamide (AM251) and 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl(4-methoxyphenyl)-methanone (AM630) were purchased from Tocris (Bristol, UK). WIN55,212-2, AM251, AM630, and naloxone were dissolved in the vehicle (a 1:1:18 ratio of cremophor: dimethylsulfoxide:saline solution) before injection. All other drugs were dissolved in sterilized distilled saline and stored at −20°C.

**Implantation of Cannula into Lateral Ventricle.** Surgical implantation of the cannula was conducted in an aseptic environment, as described earlier (Fang et al., 2012). Mice were anesthetized with pentobarbital sodium (80 mg/kg i.p.) and placed in a stereotaxic apparatus. The incision area of the scalp was shaved, and a sagittal incision was made in the midline, exposing the surface of the skull. A single hole was drilled through the targeted skull. The coordinates for the placement of the cannula were as follows: 3 mm posterior from the bregma, 1 mm lateral, and 3 mm ventral from the skull surface for the lateral ventricle (intracerebroventricular injection). To prevent occlusion, a dummy cannula was inserted into the guide cannula. The dummy cannula protruded 0.5 mm from the guide cannula. Dental cement was used to fix the guide cannula to the skull. After surgery, the animals were allowed to recover for at least 4 days; during this time, mice were gently handled daily to minimize the stress associated with manipulation of the animals throughout the experiments.

At the end of the experiments, mice were injected with methylene blue dye (3 $\mu$l), which was allowed to diffuse for 10 minutes. Mice then were decapitated, and their brains were removed and frozen. Gross dissection of the brains was used to verify the placement of the cannula. Only the data from those animals with dispersion of the dye throughout the ventricles were used in the study. The cannulation success rate was more than 95% in the present studies.

**Drug Administration.** Drugs were administered into the lateral ventricle at a fixed volume of 4 $\mu$l (at a constant rate of 10 $\mu$l/min), which was followed by 1 $\mu$l of saline to flush in the drug using a 25-$\mu$l microsyringe.

The intrathecal injection procedure was adopted as described by Hylden and Wilcox (1980). In brief, a 28-gauge needle connected to a 25-$\mu$l microsyringe was directly inserted between the $L_5$ and $L_6$ segment in mice. Puncture of the dura was indicated by a reflexive lateral flick of the tail or formation of an "S" shape by the tail (Fairbanks, 2003). Drugs were injected into subarachnoid space in a volume of 5 $\mu$l, and the catheter was also flushed with 1 $\mu$l of saline (at a constant rate of 3 $\mu$l/s).

**Nociceptive Tests.** The nociceptive response was assessed by the radiant heat tail-flick test. In brief, male Kunming mice weighing

22–25 g were used. The animals were gently restrained by hand, and a light beam was focused onto the tail. At the beginning of the study, the lamp intensity was adjusted to elicit a response in control animals within 3–5 seconds. A cutoff time was set at 10 seconds to minimize tissue damage. Tail-flick time was determined before injection and then at 5, 10, 15, 20, 30, 45, and 60 minutes postinjection. Each male mouse was used only once. Data were expressed as the percent maximum possible effect (MPE) calculated as follows: MPE (%) = 100 × [(postdrug response − baseline response)/(cutoff response − baseline response)]. The raw data from nociceptive assays were converted to area under the curve (AUC). The area under the curve depicting total %MPE versus time was computed by trapezoidal approximation over a period of 0–60 minutes. Data were statistically compared by means of one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test or Bonferroni's post-hoc test performed on AUC data.

**Body Temperature Measurement.** The male mice were placed in the specially designed restraining device as described by Rosow et al. (1980), with their tails taped lightly to horizontal posts. Each animal was used only once. The ambient temperature was regulated to 21 ± 0.5°C. The experiments were performed between 10:00 AM and 2:00 PM. Rectal temperature was measured with a thermistor probe (Machine Equipment Corporation of GaoBeiDian, Hebei, China) inserted to a depth of 2.5 cm into the rectum, which was linked to a recorder system (model BL-420E$^+$; Taimeng Technology Corporation of Chengdu, Chengdu, China). Body temperature was recorded before injection and then at 10, 20, 30, 40, 50, and 60 minutes after intracerebroventricular injection. Rectal temperature values (Δ°C) were expressed as the difference between control temperature (before injection) and temperatures after drug administration. The raw data from hypothermic assays were converted to AUC. The area under the curves depicting total Δ°C versus time were computed by trapezoidal approximation over a period of 0–60 minutes. Data were statistically compared by means of one-way ANOVA followed by Dunnett's post-hoc test performed on AUC data.

**Locomotor Activity Test.** Locomotor activity of mice was measured using the Morris Water Maze Tracking System (Taimeng Technology Corporation of Chengdu). The animals were placed individually in a Plexiglas box (50 × 50 × 30 cm) after injection of saline or drugs. Horizontal activity (distance traveled) was recorded for 15 minutes. Data obtained from the locomotor activity test were statistically compared by means of one-way ANOVA followed by Dunnett's post-hoc test.

**Tolerance Development to Antinociception.** Mice received intracerebroventricular injections of either vehicle or (m)VD-Hpα (13.4 and 20.1 nmol) once daily for 8 days. Animals were tested for tail-flick latencies before injections using the equipment and methods described earlier and then received an injection of their assigned dose of drug and were tested at 5, 10, 15, 20, and 30 minutes every testing day. The period 0–30 minutes was chosen because the maximal effects of (m)VD-Hpα were seen at the 15-minute testing time points. %MPE was calculated as the analgesia assessment described earlier. To evaluate tolerance development, Newman-Keuls post-hoc tests (paired $t$ tests) were used to compare the maximal %MPE (WIN55,212-2 at 10 minutes, and (m)VD-Hpα at 15 minutes after drug administration) obtained on days 2–8 with data from day 1.

**Place Conditioning Experiment.** The conditioned place preference (CPP) apparatus was divided into three compartments. Two identical-sized compartments (20 × 20 × 20 cm) were connected by a narrower one (5 × 20 × 20 cm). The large compartments are visually and tactually distinct (black-and-white striped walls with rough floor versus black-dotted white walls with smooth floor). These boxes could be isolated by guillotine doors.

On the preconditioning day (day 1), mice were given free access to the entire apparatus for 15 minutes, and the time spent in each compartment was measured. Mice that spent more than 60% of the time in the same compartment were excluded from the tests. On the conditioning days, mice were intracerebroventricularly injected with saline and confined to one of the compartments for 15 minutes. Approximately 6 hours later, animals were administered intracerebroventricularly saline or (m)VD-Hpα and confined to the opposite compartment. This conditioning procedure was carried out for a total of three identical conditioning sessions (days 2–4). On the test day (postconditioning day; day 5), mice were also given free access to the entire apparatus for 15 minutes, and the time spent in each compartment was measured. CPP score was expressed as time spent in the drug-associated compartment on the postconditioning day minus time spent in the drug-associated compartment on the preconditioning day. Data obtained from the CPP test were statistically compared by means of one-way ANOVA followed by the Tukey HSD (Honestly Significant Difference) test.

**Food Intake.** Mice were isolated in individual cages (24 × 15 × 9 cm). To make them accustomed to the experimental conditions, mice were given free access to water and the pellets of food for 3 days before testing days. The day of testing, at 8:00 AM, unconsumed food was removed and mice were fasted for 1 hour. After intracerebroventricular administration (10 minutes before testing), mice had access to a weighed food pellet (~4 g) laid down on the floor of the cage. At 1, 2, and 4 hours after refeeding, the pellet was briefly (<1 minute) removed and weighed. Food intake was expressed as the consumption of food pellet during the first (0–1 hour), second (1–2 hours), and third (2–4 hours) period of testing. Data obtained from the food intake test were statistically compared by means of one-way ANOVA followed by Dunnett's post-hoc test.

**Statistical Analysis.** Data were given as means ± S.E.M. Probabilities of less than 5% ($P < 0.05$) were considered statistically significant. The dose that elicits 50% efficacy ($EC_{50}$) and the corresponding 95% confidence interval were determined using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

## Results

**Antinociceptive Effects of (m)VD-Hpα.** To evaluate the antinociceptive properties of the peptide agonist (m)VD-Hpα, the antinociceptive effects of the agonist injected in supraspinal and spinal routes were investigated using the mouse tail-flick test. When administered intracerebroventricularly, (m)VD-Hpα produced a time- and dose-dependent antinociception with an $EC_{50}$ value (and 95% confidence limits) of 6.69 (5.76–7.78) nmol and a time to peak effect of 15 minutes (Fig. 1A; $F_{4,\ 38} = 685.9$, $P < 0.0001$). In addition, when given intrathecally, (m)VD-Hpα was potent in producing antinociception with an $EC_{50}$ value of 2.88 (2.60–3.18) nmol and a time to peak effect of 10 minutes (Fig. 1B; $F_{4,\ 39} = 470.3$, $P < 0.0001$).

Furthermore, to characterize the central antinociception of (m)VD-Hpα, antagonists of cannabinoid and opioid receptors were further used in the present study. The centrally active agonist WIN55,212-2 was used as a reference compound to show established effects of $CB_1$ receptor activation on antinociception. As shown in Fig. 2, coinjection of the $CB_1$ receptor antagonist AM251 (20 nmol i.c.v.) completely blocked the supraspinal antinociception induced by the cannabinoid agonist WIN55,212-2 ($F_{3,\ 29} = 117.9$, $P < 0.0001$). In contrast, neither the $CB_2$ receptor antagonist AM630 (20 nmol i.c.v.) nor the opioid receptor antagonist naloxone (5 nmol i.c.v.) altered supraspinal antinociception of WIN55,212-2 (22.5 nmol i.c.v.). In addition, at the same doses, these three antagonists did not modify the tail-flick latency in mice when administered intracerebroventricularly alone (data not shown). In mice treated with AM630 and naloxone before (m)VD-Hpα, the antinociceptive effect was still observed; however, AM251 significantly reduced the supraspinal antinociception of (m)VD-Hpα ($F_{4,\ 34} = 1198.2$, $P < 0.0001$).

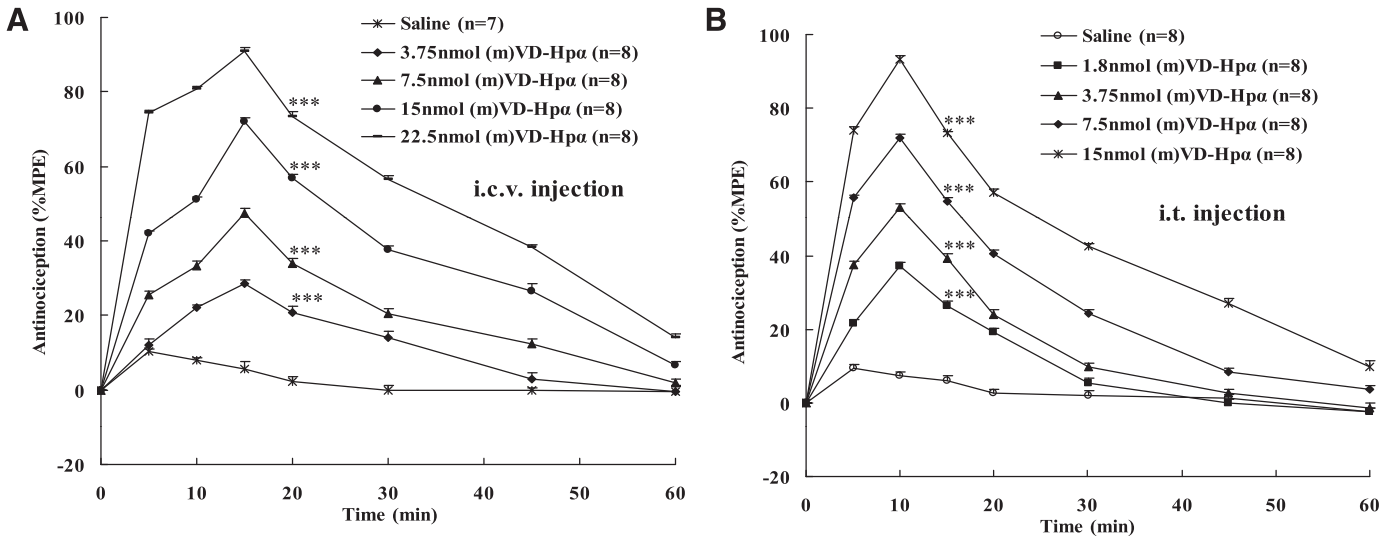


**Fig. 1.** Dose- and time-related analgesic effects of intracerebroventricularly (A) or intrathecally (B) administered (m)VD-Hpα in the mouse tail-flick assay. Data points represent means ± S.E.M. AUCs calculated 0–60 minutes from these data are statistically analyzed and are presented in the text. ***$P < 0.001$ versus saline according to one-way ANOVA followed by Dunnett's post-hoc test performed on AUC data.

At the spinal level, the antinociceptive effects induced by both WIN55,212-2 and (m)VD-Hpα were completely blocked by intrathecally coadministered AM251 ($F_{3, 29} = 118.2$, $F_{4, 34} = 548.6$, $P < 0.0001$) but not AM630 or naloxone (Fig. 3). In addition, at the same doses, intrathecal administration of these antagonists alone did not modify the tail-flick latency in mice (data not shown).

**Effects of Intracerebroventricularly Administered (m)VD-Hpα on Body Temperature and Locomotor Activity.** Furthermore, the CNS side effects of (m)VD-Hpα at its analgesic doses were investigated in a series of in vivo assays. In Fig. 4, at the antinociceptive doses (1 × $EC_{50}$, 2 × $EC_{50}$, and 3 × $EC_{50}$), the cannabinoid agonist–like profiles of (m)VD-Hpα were investigated. As shown in Fig. 4A, after intracerebroventricular administration, (m)VD-Hpα evoked a dose-related hypothermia compared with the saline group ($F_{3, 35} = 10.8$, $P < 0.0001$). The decrease in body temperature induced by (m)VD-Hpα was maximal at 10 minutes and returned to baseline value at 40 minutes after injection. At the doses of 2 × $EC_{50}$ and 3 × $EC_{50}$, (m)VD-Hpα produced marked hypothermia with a maximal effect of −1.07 ± 0.12 and −1.64 ± 0.22°C, respectively.

Locomotor activity in mice was evaluated by automatically recording distance traveled in an open field. In Fig. 4B, at the lower doses (1 × $EC_{50}$ and 2 × $EC_{50}$ intracerebroventricularly), (m)VD-Hpα did not induce a significant hypoactivity compared with saline-treated mice. However, it caused a significant decrease in locomotor activity at the 3 × $EC_{50}$ dose ($F_{3, 35} = 14.7$, $P < 0.0001$).

**Effects of Repeated Administration of (m)VD-Hpα on Tolerance Development to Thermal Antinociception.** The effects of (m)VD-Hpα (2 × $EC_{50}$ and 3 × $EC_{50}$ intracerebroventricularly) on central antinociception across repeated test days are shown in Fig. 5. On test days 1–8, (m)VD-Hpα

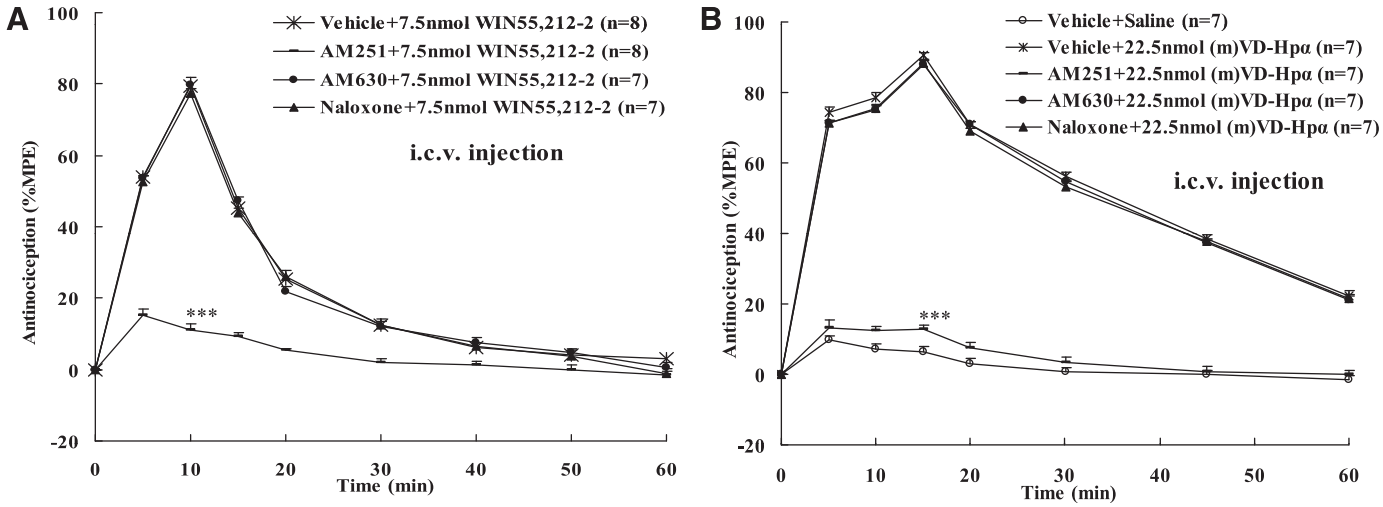


**Fig. 2.** Time course of the effects of intracerebroventricular injection of AM251 (20 nmol), AM630 (20 nmol), and naloxone (5 nmol) on the central antinociception produced by WIN55,212-2 (A) or (m)VD-Hpα (B) in the mouse tail-flick assay. Data points represent means ± S.E.M. AUCs calculated 0–60 minutes from these data are used for statistical analysis. ***$P < 0.001$ versus vehicle + agonists–injected group, according to one-way ANOVA followed by Bonferroni's post-hoc test performed on AUC data.

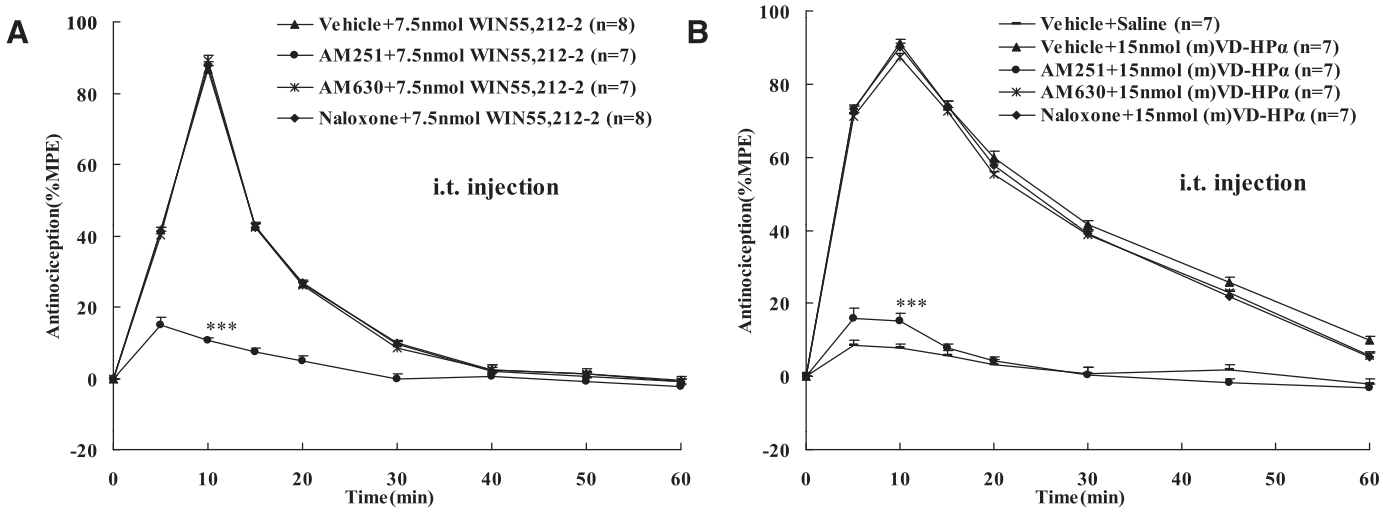


**Fig. 3.** Time course of the effects of intrathecal injection of AM251 (20 nmol), AM630 (20 nmol), and naloxone (5 nmol) on the central antinociception produced by WIN55,212-2 (A) or (m)VD-Hp$\alpha$ (B) in the mouse tail-flick assay. Data points represent means ± S.E.M. AUCs calculated 0–60 minutes from these data are used for statistical analysis. ***$P < 0.001$ versus vehicle + agonists–injected group, according to one-way ANOVA followed by Bonferroni's post-hoc test performed on AUC data.

caused an increase in tail-flick latency that was significantly greater than that observed in the saline group. Compared with day 1 (91.15 ± 1.49 %MPE), 9 nmol WIN55,212-2 produced a marked decrease in %MPE on day 5 ($F_{7,\ 63}$ = 141.4; $P <$ 0.0001), indicating that tolerance develops to central antinociception of WIN55,212-2. Similar to WIN55,212-2, compared with day 1 (73.48 ± 0.94 and 88.38 ± 1.06 %MPE), (m)VD-Hp$\alpha$ also produced a significant decrease in %MPE on day 5 ($F_{7,\ 63}$ = 188.4 and 158.6, respectively; $P < 0.0001$), indicating that tolerance develops to (m)VD-Hp$\alpha$–induced antinociception at the supraspinal level. However, it is notable that WIN55,212-2, 2 × $EC_{50}$, and 3 × $EC_{50}$ (m)VD-Hp$\alpha$ still induced 38.75 ± 1.09, 32.26 ± 1.33, and 45.63 ± 1.88 %MPE antinociception on day 8, respectively.

**Effects of Intracerebroventricularly Administered (m)VD-Hp$\alpha$ on Place Conditioning.** The effect of (m)VD-Hp$\alpha$ on place conditioning is shown in Fig. 6. Saline given intracerebroventricularly did not significantly induce the place preference change, indicating that central injections were not aversive or rewarding in the unbiased balanced paradigm of conditioned place preference in mice. Compared with saline vehicle-treated animals, intracerebroventricular injection of (m)VD-Hp$\alpha$ (1 × $EC_{50}$, 2 × $EC_{50}$, and 3 × $EC_{50}$) exerted dose-dependent conditioned place aversion in mice ($F_{3,\ 34}$ = 17.6, $P < 0.0001$).

**Effects of Intracerebroventricularly Administered (m)VD-Hp$\alpha$ on Food Consumption.** Previous reports indicated that cannabinoid agonists could also induce behavioral

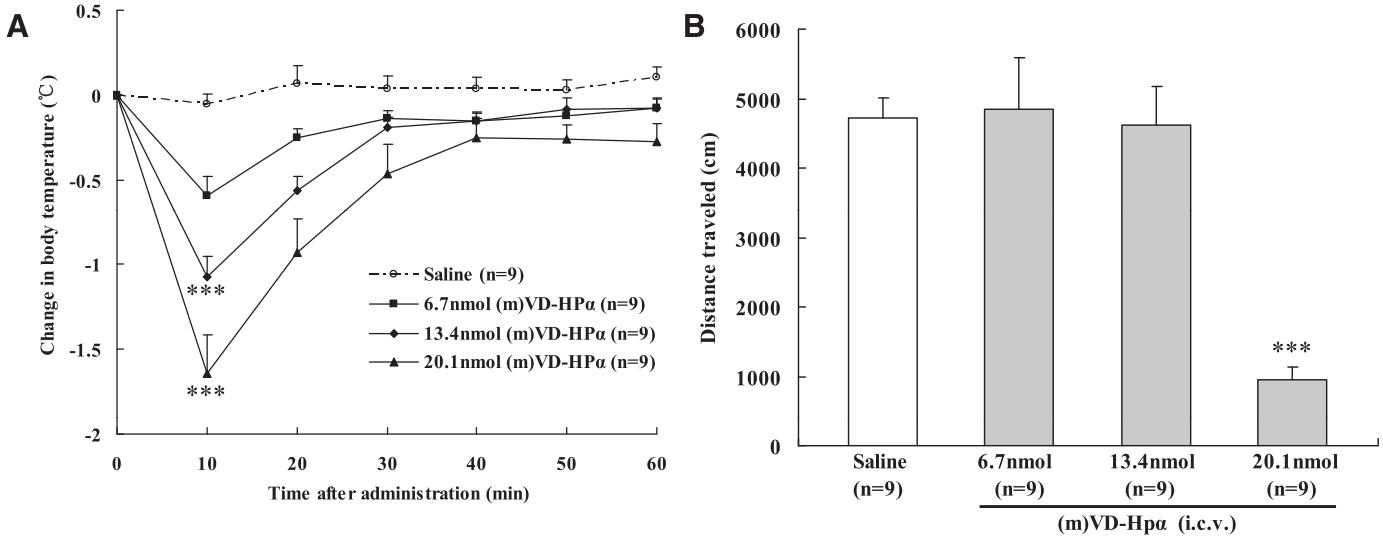


**Fig. 4.** CNS cannabinoid effects of intracerebroventricular injection of (m)VD-Hp$\alpha$ in mice. (A) Dose- and time-related hypothermic effects of intracerebroventricular administration of (m)VD-Hp$\alpha$ (6.7, 13.4, and 20.1 nmol). The value of baseline temperature before injection was 38.44 ± 0.05°C ($n$ = 36). AUCs calculated 0–60 minutes from these data are statistically analyzed with one-way ANOVA followed by Dunnett's post-hoc test performed on AUC data. (B) Locomotor activities of intracerebroventricular administration of (m)VD-Hp$\alpha$ (6.7, 13.4, and 20.1 nmol). Data points represent means ± S.E.M. from experiments conducted on 9 mice. ***$P < 0.001$ versus saline according to one-way ANOVA followed by Dunnett's post-hoc test.

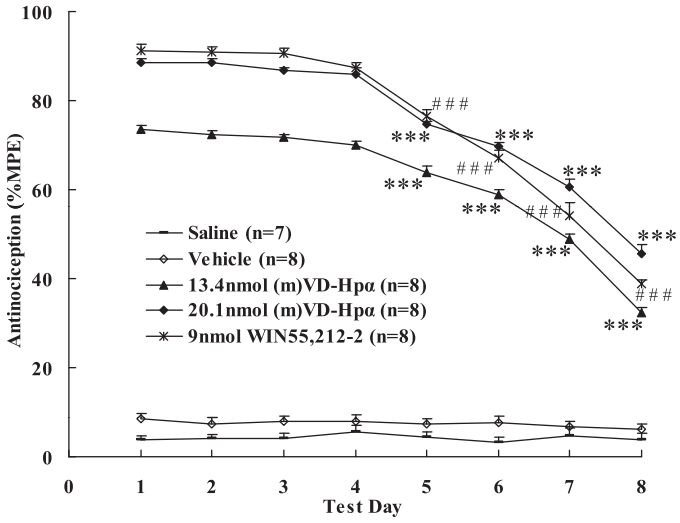


**Fig. 5.** Antinociceptive tolerance studies of intracerebroventricular injection of (m)VD-Hpα (13.4 and 20.1 nmol) and WIN55,212-2 (9 nmol). Animals received intracerebroventricular injections of saline, vehicle, (m)VD-Hpα, or WIN55,212-2 once daily for 8 days. Analgesia was measured each day for 30 minutes after drug injection using the tail-flick assay. Ordinal values represent the daily analgesic response, as expressed by the maximal %MPE [WIN55,212-2 at 10 minutes and (m)VD-Hpα at 15 minutes] after injection. Data points represent the mean ± S.E.M. Tolerance to the analgesic effects of (m)VD-Hpα and WIN55,212-2 begins to develop on day 5 [$^{***}P < 0.001$ versus effect of (m)VD-Hpα on day 1, and $^{\#\#\#}P < 0.001$ versus effect of WIN55,212-2 on day 1, according to Newman-Keuls post-hoc tests).

effects on appetite. The effect of (m)VD-Hpα on food intake is shown in Fig. 7. Compared with the saline group, intracerebroventricular injection of (m)VD-Hpα ($1 \times EC_{50}$, $2 \times EC_{50}$, and $3 \times EC_{50}$) caused a dose-dependent increase in food intake during the second (1–2 hours) and third (2–4 hours) period of testing ($F_{3, 49} = 6.3$ and 4.4, respectively; $P < 0.01$). However, (m)VD-Hpα induced a slight, but not statistically significant, increase in food intake during the first (0–1 hour) period ($F_{3, 49} = 0.87$; $P = 0.46$).

## Discussion

The endocannabinoid system is considered an attractive target for pharmaceutical development (Hutcheson et al., 1998; Richardson, 2000; Bushlin et al., 2010). The endocannabinoid receptors have been traditionally thought to act through the effects of the lipid endocannabinoids (Hutcheson et al., 1998; Richardson, 2000; Bushlin et al., 2010). Interestingly, the recent studies indicated that cannabinoid receptors were recognized by hemopressin-related peptides with affinities in the nanomolar range (Heimann et al., 2007; Gomes et al., 2009). (m)RVD-Hpα and (m)VD-Hpα were reported to function as a novel endogenous peptide agonist of the $CB_1$ receptor in vitro (Gomes et al., 2009). However, the data obtained from studies in the phosphorylation level of extracellular signal-regulated kinase 1/2 or the release of $G\alpha_{i16}$-facilitated $Ca^{2+}$ demonstrated that the signal transduction pathway of (m)RVD-Hpα was distinct from that activated by 2-arachidonoylglycerol and Hu-210 (Gomes et al., 2009). Thus, this is the first study to demonstrate that (m)VD-Hpα exerted central antinociception via the $CB_1$ receptor and exhibited various CNS effects at the supraspinal level in a manner similar to the cannabinoid agonists.

Cannabinoids were reported to exert their antinociceptive effects via complex mechanisms at peripheral, spinal, and supraspinal levels (Lichtman and Martin, 1991; Richardson, 2000; Pertwee, 2001; Guindon and Beaulieu, 2009). In the present study, the antinociceptive properties of (m)VD-Hpα were investigated in the mouse tail-flick test. As expected, intracerebroventricular injection of (m)VD-Hpα dose-dependently caused acute antinociception. Moreover, (m)VD-Hpα–induced supraspinal antinociception was significantly attenuated by the

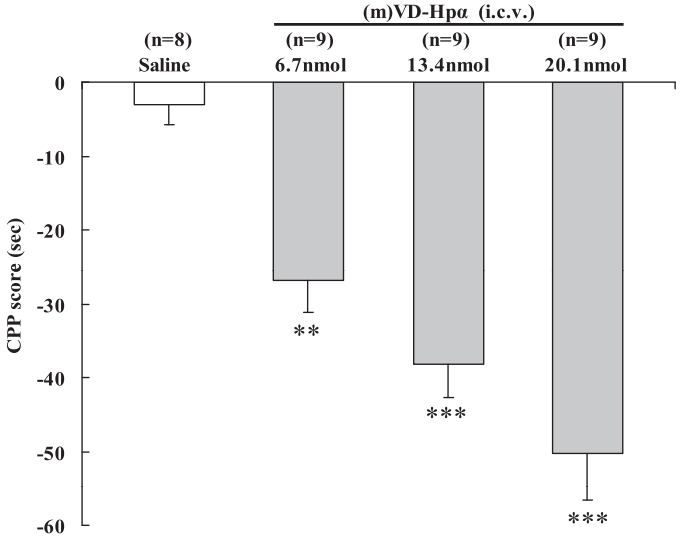


**Fig. 6.** Place conditioning induced by (m)VD-Hpα given intracerebroventricularly in mice. CPP score was expressed as time spent in the drug-associated compartment on the postconditioning day minus time spent in the drug-associated compartment on the preconditioning day during a period of 15 minutes. Results are presented as the mean ± S.E.M. from experiments conducted on 8–9 mice. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus saline according to one-way ANOVA followed by the Tukey HSD (Honestly Significant Difference) test.

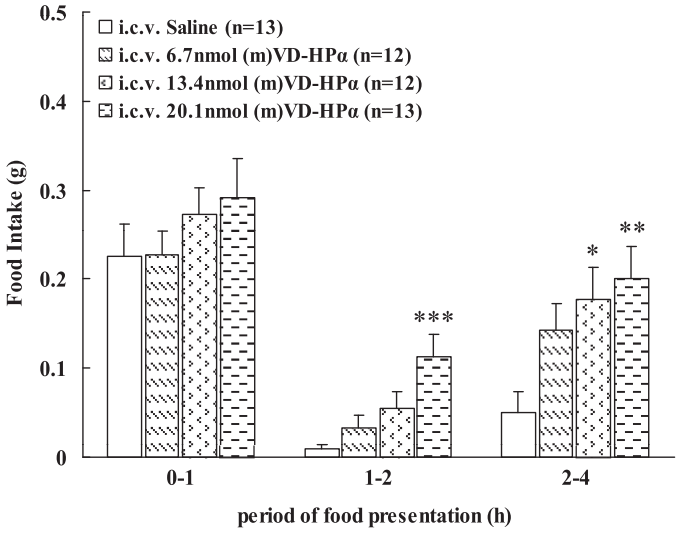


**Fig. 7.** Effects of (m)VD-Hpα on food intake in mice. Mice were administered intracerebroventricularly with saline or (m)VD-Hpα (6.7, 13.4, and 20.1 nmol). Ten minutes after injection, the animals were refed, and food consumption was measured during the periods indicated. Results are presented as the mean ± S.E.M. from experiments conducted on 12–13 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus saline according to one-way ANOVA followed by Dunnett's post-hoc test.

$CB_1$ receptor antagonist AM251 but not by the $CB_2$ receptor antagonist AM630. Likewise, our present results suggested that (m)VD-Hp$\alpha$ also produced marked antinociception at the spinal level, which was mediated by activation of the $CB_1$ receptor but not the $CB_2$ receptor. Taken together, the novel endogenous $CB_1$ receptor agonist (m)VD-Hp$\alpha$ can act at both the supraspinal and spinal sites to decrease nociceptive responses via the $CB_1$ receptor pathway, which is consistent with previous reports that the antinociceptive effects of centrally administered cannabinoid agonists $\Delta^9$-tetrahydrocannabinol, CP55,940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol] and WIN55,212-2 were sensitive to the selective $CB_1$ receptor antagonists (Welch et al., 1998; Richardson, 2000; Fang et al., 2012).

Recently, the data obtained from the interaction of the cannabinoid and opioid system have suggested that endogenous opioids might be involved in the pain regulation of cannabinoids (Bushlin et al., 2010; Parolaro et al., 2010). Reports have also revealed that the antinociception of $\Delta^9$-tetrahydrocannabinol was prevented by the general opioid antagonist naloxone (Reche et al., 1996; Bushlin et al., 2010). However, our data indicated that the central antinociception of the novel agonist (m)VD-Hp$\alpha$ was independent of the opioid system. In agreement with these observations, our previous results also demonstrated that the opioid system was not involved in the central antinociception of the cannabinoid agonist WIN55,212-2 (Fang et al., 2012).

The $CB_1$ receptor is predominantly located in the CNS (Pacher et al., 2006; Guindon and Beaulieu, 2009). In theory, central administration of cannabinoid $CB_1$ agonists not only produced analgesic effects, but also caused a number of undesirable side effects. However, a high dose of hemopressin did not impair motor activity or alter pentobarbital-induced sleeping time, indicating the absence of unwanted motor or sedative side effects (Heimann et al., 2007). To further characterize the profiles of the endogenous $CB_1$ agonist (m)VD-Hp$\alpha$, the present work was designed to evaluate its CNS side effects on hypothermia, hypoactivity, reward, and antinociception tolerance development.

Previous studies have shown that treatment with the classic agonists of cannabinoid receptors can induce a series of behavioral responses, such as antinociception, hypothermia, suppression of activity, and immobility (Richardson, 2000; Pacher et al., 2006). Our data also indicated that intracerebroventricular administration of (m)VD-Hp$\alpha$ significantly decreased rectal temperature, and the highly antinociceptive dose ($3 \times EC_{50}$) of (m)VD-Hp$\alpha$ markedly suppressed locomotor activity. These results demonstrated that (m)VD-Hp$\alpha$ also exerted an agonist-like profile of effects on hypothermia and hypoactivity, which further supported a cannabinoid agonist character of (m)VD-Hp$\alpha$.

In addition, cannabinoid agonists also have a well known propensity to induce tolerance development and reward (Hutcheson et al., 1998; De Vry et al., 2004; Pacher et al., 2006; Bushlin et al., 2010; Blankman and Cravatt, 2013; Mechoulam and Parker, 2013). Thus, we focused our further studies on these undesirable side effects of (m)VD-Hp$\alpha$ after supraspinal administration. In tolerance development assay, (m)VD-Hp$\alpha$ produced a significant, albeit not dramatic, decrease of antinociception in a manner similar to the classic agonist WIN55,212-2.

It is well known that drugs that activated reward system must be evaluated for abuse potential. However, cannabinoids exerted complex modulating effects in the place-conditioning paradigm (Pacher et al., 2006; Mechoulam and Parker, 2013). Cannabinoids generally produce aversive-like responses. In contrast, some research groups have independently reported that cannabinoids induce robust CPP. The crucial differences might be related to cannabinoid doses, timings, and potencies (Maldonado, 2002; Gardner, 2005; Pacher et al., 2006; Bushlin et al., 2010; Panlilio et al., 2010; Mechoulam and Parker, 2013). In addition, the lipid-based endocannabinoid anandamide does not induce any behavioral response on the place-conditioning paradigm (Maldonado, 2002; Gardner, 2005; Pacher et al., 2006; Bushlin et al., 2010; Panlilio et al., 2010; Mechoulam and Parker, 2013). Our rewarding data showed that central administration of (m)VD-Hp$\alpha$ produced a dose-related conditioned place aversion, suggesting that activation of the $CB_1$ receptor in the brain may result in conditioned place aversion. Moreover, conditioned place aversion induced by other cannabinoid agonists was reported to be abolished by pretreatment with the $CB_1$ receptor antagonist SR141716A [*N*-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] (Maldonado, 2002; Gardner, 2005). Therefore, the data on the conditioned place aversion of (m)VD-Hp$\alpha$ implied that the $CB_1$ receptor could be an attractive therapeutic target for analgesics without drug-seeking behavior.

Collectively, (m)VD-Hp$\alpha$ significantly produced $CB_1$-mediated antinociceptive activities at the supraspinal and spinal level, and the $EC_{50}$ values were 6.69 and 2.88 nmol, respectively. Furthermore, the present work also indicated that, at potently analgesic doses, (m)VD-Hp$\alpha$ induced hypothermia, hypoactivity, tolerance development, and conditioned place aversion at the supraspinal level. However, it is notable that the cannabinoid agonist–like profiles of (m)VD-Hp$\alpha$ at lower antinociceptive doses ($1 \times EC_{50}$ and $2 \times EC_{50}$ intracerebroventricularly) were absent or weak in mice, which implied that the use of a suitable dosage of the drug might provide an effective approach to minimize the CNS side effects of (m)VD-Hp$\alpha$ or separate its antinociception from its side effects.

In addition, the cannabinoids system also plays an important role in appetite regulation. Both $\Delta^9$-tetrahydrocannabinol and anandamide increased food intake via the $CB_1$ receptor (Pacher et al., 2006). Hemopressin, a selective inverse agonist of the $CB_1$ receptor, was recently reported to inhibit food intake in both normal and obese rodent models, and block $CB_1$ agonist–induced hyperphagia in vivo (Dodd et al., 2010; Bomar and Galande, 2013). The recent studies also demonstrated that the peptide hemopressin modulated the function of key feeding-related brain nuclei (Dodd et al., 2013). Our data indicated that central injection of (m)VD-Hp$\alpha$ dose-dependently stimulated food consumption in mice, which further suggested that (m)VD-Hp$\alpha$ acted as a selective agonist of the $CB_1$ receptor.

In conclusion, the present work demonstrates that the novel cannabinoid peptide agonist (m)VD-Hp$\alpha$ induces $CB_1$-mediated central antinociception with some CNS effects, which further supports a $CB_1$ agonist character of (m)VD-Hp$\alpha$. In addition, the current study will be helpful to understand the in vivo properties of the endogenous peptide agonist of the cannabinoid $CB_1$ receptor. Furthermore, it is notable that the chemical structure of (m)VD-Hp$\alpha$ is different from that of other cannabinoid agonists. It is expected that (m)VD-Hp$\alpha$ may play a broader role in pharmacological characterization of the cannabinoids system, especially for the study of the $CB_1$ receptor.

### Authorship Contributions

*Participated in research design:* R. Wang, Fang.

*Conducted experiments:* Han, Z.-L. Wang, X.-H. Li, N. Li, Chang, Pan, Tang.

*Performed data analysis:* Han, Fang.

*Wrote or contributed to the writing of the manuscript:* R. Wang, Fang, Han.

### References


Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, Rubino T, Michalski CW, Marsicano G, and Monory K, et al. (2007) Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci* **10**:870–879.

Blais PA, Côté J, Morin J, Larouche A, Gendron G, Fortier A, Regoli D, Neugebauer W, and Gobeil F, Jr (2005) Hypotensive effects of hemopressin and bradykinin in rabbits, rats and mice. A comparative study. *Peptides* **26**:1317–1322.

Blankman JL and Cravatt BF (2013) Chemical probes of endocannabinoid metabolism. *Pharmacol Rev* **65**:849–871.

Bomar MG and Galande AK (2013) Modulation of the cannabinoid receptors by hemopressin peptides. *Life Sci* **92**:520–524.

Bushlin I, Rozenfeld R, and Devi LA (2010) Cannabinoid-opioid interactions during neuropathic pain and analgesia. *Curr Opin Pharmacol* **10**:80–86.

Dale CS, Pagano RdeL, Rioli V, Hyslop S, Giorgi R, and Ferro ES (2005) Antinociceptive action of hemopressin in experimental hyperalgesia. *Peptides* **26**: 431–436.

De Vry J, Jentzsch KR, Kuhl E, and Eckel G (2004) Behavioral effects of cannabinoids show differential sensitivity to cannabinoid receptor blockade and tolerance development. *Behav Pharmacol* **15**:1–12.

Dodd GT, Mancini G, Lutz B, and Luckman SM (2010) The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. *J Neurosci* **30**:7369–7376.

Dodd GT, Worth AA, Hodkinson DJ, Srivastava RK, Lutz B, Williams SR, and Luckman SM (2013) Central functional response to the novel peptide cannabinoid, hemopressin. *Neuropharmacology* **71**:27–36.

Fairbanks CA (2003) Spinal delivery of analgesics in experimental models of pain and analgesia. *Adv Drug Deliv Rev* **55**:1007–1041.

Fang Q, Han ZL, Li N, Wang ZL, He N, and Wang R (2012) Effects of neuropeptide FF system on $CB_1$ and $CB_2$ receptors mediated antinociception in mice. *Neuropharmacology* **62**:855–864.

Gardner EL (2005) Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacol Biochem Behav* **81**:263–284.

Gomes I, Grushko JS, Golebiewska U, Hoogendoorn S, Gupta A, Heimann AS, Ferro ES, Scarlata S, Fricker LD, and Devi LA (2009) Novel endogenous peptide agonists of cannabinoid receptors. *FASEB J* **23**:3020–3029.

Guindon J and Beaulieu P (2009) The role of the endogenous cannabinoid system in peripheral analgesia. *Curr Mol Pharmacol* **2**:134–139.

Heimann AS, Gomes I, Dale CS, Pagano RL, Gupta A, de Souza LL, Luchessi AD, Castro LM, Giorgi R, and Rioli V, et al. (2007) Hemopressin is an inverse agonist of CB1 cannabinoid receptors. *Proc Natl Acad Sci USA* **104**:20588–20593.

Hutcheson DM, Tzavara ET, Smadja C, Valjent E, Roques BP, Hanoune J, and Maldonado R (1998) Behavioural and biochemical evidence for signs of abstinence in mice chronically treated with delta-9-tetrahydrocannabinol. *Br J Pharmacol* **125**:1567–1577.

Hylden JL and Wilcox GL (1980) Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* **67**:313–316.

Ibrahim MM, Rude ML, Stagg NJ, Mata HP, Lai J, Vanderah TW, Porreca F, Buckley NE, Makriyannis A, and Malan TP, Jr (2006) CB2 cannabinoid receptor mediation of antinociception. *Pain* **122**:36–42.

Lichtman AH and Martin BR (1991) Spinal and supraspinal components of cannabinoid-induced antinociception. *J Pharmacol Exp Ther* **258**:517–523.

Maldonado R (2002) Study of cannabinoid dependence in animals. *Pharmacol Ther* **95**:153–164.

Mechoulam R and Parker LA (2013) The endocannabinoid system and the brain. *Annu Rev Psychol* **64**:21–47.

Pacher P, Bátkai S, and Kunos G (2006) The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* **58**:389–462.

Panlilio LV, Justinova Z, and Goldberg SR (2010) Animal models of cannabinoid reward. *Br J Pharmacol* **160**:499–510.

Parolaro D, Rubino T, Viganò D, Massi P, Guidali C, and Realini N (2010) Cellular mechanisms underlying the interaction between cannabinoid and opioid system. *Curr Drug Targets* **11**:393–405.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74**:129–180.

Pertwee RG (2001) Cannabinoid receptors and pain. *Prog Neurobiol* **63**:569–611.

Reche I, Fuentes JA, and Ruiz-Gayo M (1996) A role for central cannabinoid and opioid systems in peripheral delta 9-tetrahydrocannabinol-induced analgesia in mice. *Eur J Pharmacol* **301**:75–81.

Richardson JD (2000) Cannabinoids modulate pain by multiple mechanisms of action. *J Pain* **1**:2–14.

Rioli V, Gozzo FC, Heimann AS, Linardi A, Krieger JE, Shida CS, Almeida PC, Hyslop S, Eberlin MN, and Ferro ES (2003) Novel natural peptide substrates for endopeptidase 24.15, neurolysin, and angiotensin-converting enzyme. *J Biol Chem* **278**:8547–8555.

Rosow CEMJ, Miller JM, Pelikan EW, and Cochin J (1980) Opiates and thermoregulation in mice. I. Agonists. *J Pharmacol Exp Ther* **213**:273–283.

Welch SP, Huffman JW, and Lowe J (1998) Differential blockade of the antinociceptive effects of centrally administered cannabinoids by SR141716A. *J Pharmacol Exp Ther* **286**:1301–1308.


**Address correspondence to:** Dr. Rui Wang, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 199 Donggang West Road, Lanzhou, 730000, People's Republic of China. E-mail: wangrui@lzu.edu.cn