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Centrally mediated antinociceptive effects of cannabinoid receptor ligands in rat models of nociception

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ABSTRACT

The endogenous nonapeptide hemopressin (HE) demonstrates potent block of the cannabinoid subtype-1 (CB1) receptor in vitro and robust antinociception in vivo. The current study evaluated the effects of centrally administered HE in mechanistically distinct pre-clinical rat models of pain—the hot plate test and the hind paw formalin test. The non-subtype selective CB receptor agonist WIN 55,212-2 was tested concurrently as a positive control. In the hot plate test, neither intrathecal (i.t.) HE nor WIN 55,212-2 significantly altered the latency to respond to noxious heat. By contrast, i.t. HE and WIN 55,212-2 significantly reduced pain-related behaviors in the formalin test. Possible HE functionality as a CB1 receptor antagonist at the spinal level was evaluated in the formalin test. Intrathecal pretreatment with HE did not attenuate the antinociceptive effect of i.t. WIN 55,212-2. However, pretreatment with the CB1 receptor antagonist rimonabant did; i.t. rimonabant pretreatment was not antinociceptive. Potential supraspinal antinociceptive activity of HE was also evaluated. Whereas intracerebroventricular (i.c.v.) injection of WIN 55,212-2 reduced pain-related behaviors in the formalin test, interestingly, i.c.v. HE increased behaviors. In the current study, an antinociceptive effect with the CB receptor ligand HE was obtained under the specific condition of tissue injury and not in the uninjured state. Thus, HE could be a useful analgesic peptide with a novel spinal mechanism of action.

1. Introduction

Although the use of *Cannabis sativa* for the treatment of various neurological disorders, including chronic pain, is supported by clinical data, adverse effects, such as cognitive impairment and hallucination, limit its widespread clinical use (Iskedjian et al., 2007; Russo et al., 2007). Given socio-political controversy surrounding the medical use of *C. sativa* and its bioactive CB components, as well as the side-effects, alternate CB receptor ligands are needed for clinical use.

The nonapeptide hemopressin (HE), derived from the α-chain of hemoglobin, was isolated from rat brain homogenates and demonstrated hypotensive effects in rats in vivo (Rioli et al., 2003). Hemopressin binds to rat brain cannabinoid subtype-1 (CB1) receptors with sub-nanomolar potency, which is slightly less than that of the CB1 receptor antagonist rimonabant (Heimann et al., 2007). Hemopressin blocks the effect of the CB receptor agonist HU-210 in in vitro functional assays. Furthermore, in the absence of an agonist in these assays, HE behaves as an inverse agonist, similar to rimonabant, which also demonstrates inverse agonist activity. Given these in vitro effects, it is surprising that such a peptide would demonstrate robust antinociceptive effects, which are consistently observed with CB receptor agonists. Nonetheless, carrageenan-induced hind paw hypersensitivity to noxious stimulation was markedly attenuated with intrathecal (i.t.) injection of HE (Heimann et al., 2007). No antinociceptive effect was noted in the contralateral, uninflamed paw, indicating that the effect of HE was limited to tissue injury-induced pain. Neurological responses typically associated with antinociceptive doses of CB1 receptor agonists, including hypothermia, catalepsy and hypoactivity, were not reported with antinociceptive doses of HE (Heimann et al., 2007; Martin et al., 1991). Thus, it is possible that HE induces an antinociceptive effect via a novel CB1 receptor-mediated mechanism.

An additional attractive feature of HE over a small molecule compound is that it may be possible to insert its gene into cells. Cells engineered to express analgesic substances, such as HE, implanted into the subarachnoid space could be a long-term solution to managing chronic pain (Eaton, 2006; Jeon, 2011). Long-term antinociception has been demonstrated in various animal pain models with cells that produce and release endogenous analgesic substances into the intrathecal space (Hentall and Sagen, 2000). Presently, there is limited information concerning the efficacy of i.t. HE across a range of pain models.

The primary objective of the current study was to determine the efficacy of i.t. injected HE in response to noxious thermal stimulation and in a rat pain model of peripheral, acute inflammation. Given that

Abbreviations: CB, cannabinoid; A_{50}, 50% antinociceptive dose; HE, hemopressin; i.c.v., intracerbroventricular; i.t., intrathecal; MPE, maximum possible effect.

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brain CB1 receptors also mediate the neurological effects of systemically administered CB receptor agonists, a possible antinociceptive effect of HE was also evaluated following injection into the lateral ventricle (i.c.v). The nonselective CB receptor agonist WIN 55,212-2 was used as a positive control in these tests.

A secondary objective of the current study was to evaluate a possible in vivo CB1 receptor antagonist effect of HE, since HE has been shown to potently bind to the CB1 receptor and block the effects of a CB receptor agonist in vitro. Rats were i.t. pretreated with HE and then i.t. injected with an antinociceptive dose of WIN 55,212-2 in the formalin test. As a positive control, rats were pretreated with rimonabant prior to i.t. injection of WIN 55,212-2.

2. Materials and methods

Procedures were reviewed and approved by the University of Miami Animal Care and Use Committee and followed recommendations of the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Male Sprague–Dawley rats (250–275 g; Harlan, IN) were used in these experiments. Upon arrival, rats were allowed to acclimate to the animal facility for 5–7 days prior to surgery. Rats were housed two per cage and allowed free access to food and water. Following cannulation surgeries, however, rats were singly housed. At the end of the studies, rats were euthanized with CO_2.

2.1. Surgical procedures

For all surgical procedures, aseptic surgical techniques were used, including the use of sterile instruments, gloves and personal protective equipment. Following shaving of the rat skin, the surgical area was swabbed with chlorhexidine. Rats were anesthetized and maintained on isoflurane in O_2 for the duration of the surgical procedures. Rats were allowed at least 3 days to recover from surgery prior to use in experiments.

2.1.1. Intrathecal catheters

The method of implanting an i.t. catheter in rats has been described elsewhere (Yaksh and Rudy, 1976). Briefly, rats were anesthetized and the head secured in a stereotaxic unit. The atlanto-occipital membrane was exposed and cut and an i.t. catheter (ReCathCo, Allison Park, PA) was threaded down the i.t. space. The catheter was secured to the musculature with sutures and the skin incision was closed with cyanoacrylate. After flushing the catheter with 10 μl saline, the externalized catheter was melted shut. At the end of testing, prior to euthanasia with CO_2 overdose, 5 μl of 5% lidocaine was i.t. injected to assess the location of the catheter tip. An acute bilateral flaccid paralysis of the hind limbs indicated that the catheter tip was in the correct spinal position.

2.1.2. Intracerebroventricular surgery

The method of implanting i.c.v. cannulae into the right ventricular space and the stereotaxic coordinates (Anterior–Posterior: −0.7 mm from bregma; Medial–Lateral: −1.5 mm from bregma; Dorsal–Ventral: −3.5 mm from the top of the skull) were adopted from a method described elsewhere (Taylor et al., 1994). The guide cannula was secured in place with screws and dental cement. (Cannula parts were obtained from Plastics One, Inc., Roanoke, VA. Dental cement was obtained from Stoelting, Wood Dale, IL.) A dummy cannula was inserted into the guide cannula to keep it patent. Five microliters of either drug or vehicle was injected into the ventricular space with an injection cannula that extended 1 mm below the guide cannula. At the end of the experiment, prior to euthanasia, 5 μl of methylene blue was injected into the right ventricular space to confirm proper placement of the guide cannula.

2.2. Testing procedures

2.2.1. Hot plate test

Hind paw sensitivity of rats to a noxious heat stimulus was assessed using the hot plate test. Rats were placed on a heated (54.5 °C) surface encased by a Plexiglas chamber. When the rat licked its hind paw, the rat was removed from the apparatus. The duration of time between placement of the rat on the heated surface and the hind paw lick was recorded as the response latency (in seconds). Prior to injection of either drug or vehicle, the baseline response latency was measured. Rats were tested once every 30 min up to 120 min post-injection. To prevent possible plantar skin damage, a cut-off of 45 s was used. Following completion of the test, rats were euthanized.

2.2.2. Formalin test

Ten minutes after injection (either i.t. or i.c.v.) of either drug or vehicle, 50 μl of 5% formalin was subcutaneously injected into the left plantar hind paw and rats were immediately placed in clear Plexiglas chambers (Dubuisson and Dennis, 1977). The number of hind paw flinching and licking occurring in one min were counted in 5 min intervals up to 60 min post-formalin injection (Abbott et al., 1995; Amodel and Paxinos, 1980; Tjolsen et al., 1992). Phase 1 ("acute" phase) was defined as the first min following formalin injection (0–1 min) and phase 2 ("tonic" phase) was defined as 15–61 min post-formalin injection (Hama et al., 2006; Hama and Sagen, 2009). Rats were used only once in this test.

2.2.2.1. Antagonism of intrathecal WIN 55,212-2 in the formalin test

To test for a possible CB1 receptor-mediated antagonist effect of HE on WIN 55,212-2-induced antinociception, rats were i.t. pre-treated with 1 μg HE (or vehicle), followed 10 min later by i.t. injection of 30 μg WIN 55,212-2 (or vehicle). Formalin was injected into the hind paw 10 min following the second i.t. injection. In a separate comparator group, i.t. injection of 30 μg rimonabant (or vehicle) was followed 10 min later by 30 μg WIN 55,212-2.

Thus, there were four treatment groups in the antagonist arm of the study (pre-treatment/post-treatment): *i*) vehicle/vehicle, *ii*) vehicle/WIN 55,212-2, *iii*) antagonist/vehicle and *iv*) antagonist/WIN 55,212-2.

2.3. Drugs

A volume of 5 μl was used for i.t. and i.c.v. injections of drugs. A 5 μl vehicle flush followed i.t. drug injection. Hemopressin (PVNFKFLSH) was obtained from 21st Century Biochemicals (Marlboro, MA) and dissolved in saline. WIN 55,212-2 mesylate was obtained from Sigma-Aldrich, Co. (St. Louis, MO) and was dissolved in a vehicle of 45% 2-hydroxypropyl-ß-cyclodextrin in saline. Rimonabant was obtained from Cayman Chemical, Co. (Ann Arbor, MI) and dissolved in a vehicle of 10% DMSO: 10% Tween-80: 80% saline.

In the current study, the highest tested i.t. dose of WIN 55,212-2 was 30 μg. Higher doses, e.g. 100 μg, led to hind limb flaccid paralysis and it has been reported that hind paw withdrawal thresholds are elevated well beyond normal levels (Martin et al., 1999). Doses of HE tested in the current study were based on i.t. doses used by Heimann et al. (2007). Additional doses (3, 10 μg), higher than those tested by Heimann et al. (2007) were used as well. The i.t. dose of rimonabant (30 μg) was chosen from studies that reported no observable side-effects and demonstrated in vivo antagonism of CB receptor agonists (Kang et al., 2007; Khodayar et al., 2006; Martin et al., 1999; Welch et al., 1998; Yoon and Choi, 2003).

2.4. Statistical analysis

The drug effects on behavior were converted to percentages of the maximum. For the hot plate test, response latencies were converted to percent maximum possible effect (MPE):

MPE % = ((Drug latency − Baseline latency) ÷ (45 sec − Baseline latency)) × 100.

The effects of the drugs on formalin-evoked pain-related behaviors were expressed as a percent inhibitory effect on the total behaviors in phase 1 and phase 2:

Inhibition % = (Total number of Vehicle behaviors − Total number of Drug behaviors) / (Total number of Vehicle behaviors) × 100.

Thus, 0% inhibition indicates a lack of effect on formalin-evoked behaviors, whereas 100% inhibition indicates complete suppression of behaviors.

The A_{50} (50% antinociceptive dose) and 95% confidence limits (95% C.L.) of the drugs were calculated from the linear portions of the log dose–response curves using a computer program (Tallarida and Murray, 1981). The program can be found on the Web at: http://www.u.arizona.edu/~michaelo/.

Statistical comparisons between treatment groups over time were performed using a repeated-measure two-way ANOVA with Student–Newman-Keuls Test for *post hoc* comparisons. In the formalin test, the effects of each treatment on the total pain-related behaviors in phase 1 and phase 2 were compared using one-way ANOVA with Dunnett's Test for *post hoc* comparison against the vehicle treatment. Statistical analysis of a pretreatment effect, of either HE or rimonabant, on the antinociceptive effect of WIN 55,212-2 was performed using a two-way ANOVA. Statistical significance was taken at $p<0.05$. Data are expressed as mean ± S.E.M.

3. Results

At the highest tested doses of HE, rimonabant, WIN 55,212-2 and the vehicles, no adverse side-effects, such as sedation and catalepsy, were observed following either i.t. or i.c.v. injection.

3.1. Effect of centrally administered CB receptor ligands in the hot plate test

Prior to i.t. injection, the mean response latency (±S.E.M.) of all rats was 11.7 ± 0.4 s. There was no significant change in latencies following i.t. injection of either HE (0.1, 1, 3, 10 μg), WIN 55,212-2 (3, 10, 30 μg) or rimonabant (3, 30 μg) (Fig. 1; $p>0.05$ vs. vehicle). Intrathecal vehicle injection did not affect latencies as well.

Since there was a lack of an antinociceptive effect following spinal injection of WIN 55,212-2, to confirm the presence of a centrally mediated antinociception, WIN 55,212-2 was administered i.c.v. An antinociceptive effect was observed 30 min after i.c.v. injection of 30 μg WIN 55,212-2 (Fig. 1; $p<0.05$ vs. vehicle). Intracerebroventricular injection of HE did not lead to an antinociceptive effect.

3.2. Effect of centrally administered CB receptor ligands in the formalin test

In rats i.t. treated with vehicle, the total number of pain-related behaviors in phase 1 was 18.3 ± 3.0 and 131.1 ± 10 in phase 2. Both i.t.-injected WIN 55,212-2 (Fig. 2) and HE (Fig. 3) dose-dependently decreased phase 1 behaviors ($p<0.05$ vs. vehicle). However, for 10 μg HE, there was no significant effect on pain-related behavior. For phase 1, the A_{50} (95% C.L.) for WIN 55,212-2 was 2.1 (1.1–4.1) μg. Because of the inverted U-shaped dose–response curve for HE, an estimated A_{50} was calculated, which was 0.6 μg (Fig. 4A).

In phase 2, i.t. 30 μg WIN 55,212-2 and i.t. 3 μg HE reduced phase 2 behaviors (Figs. 2, 3; $p<0.05$ vs. vehicle). The highest tested dose of HE, 10 μg, increased phase 2 behaviors above that of the vehicle-treated group, but this increase was not statistically significant ($p>0.05$ vs. vehicle). The A_{50} values for phase 2 were not calculated since the effects of the highest doses of WIN 55,212-2 and HE did not exceed 50% inhibition (Fig. 4B).

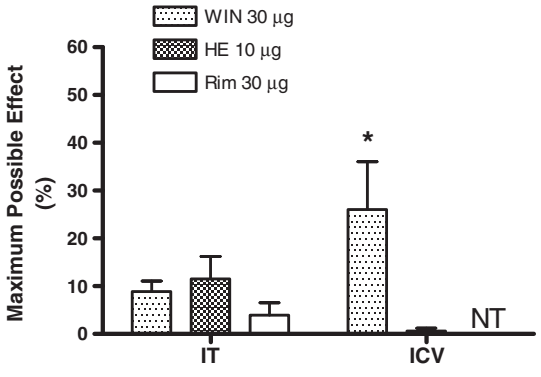

Fig. 1. Maximum possible effect of intrathecal and intracerebroventricular injection of WIN 55,212-2, hemopressin and rimonabant in the hot plate test in rats. Rats were either intrathecally (IT) or intracerebroventricularly (ICV) injected with either 30 μg WIN 55,212-2 (WIN), 10 μg hemopressin (HE), 30 μg rimonabant (Rim) or an equal volume of vehicle. The effect of the compound 30 min following injection is shown. A significant antinociceptive effect of WIN was observed 30 min following ICV injection. Vehicle injection did not significantly change withdrawal latencies (data not shown). Data are expressed as mean ± S.E.M. n = 3–8/group. *$p<0.05$ vs. Vehicle. NT, not tested.

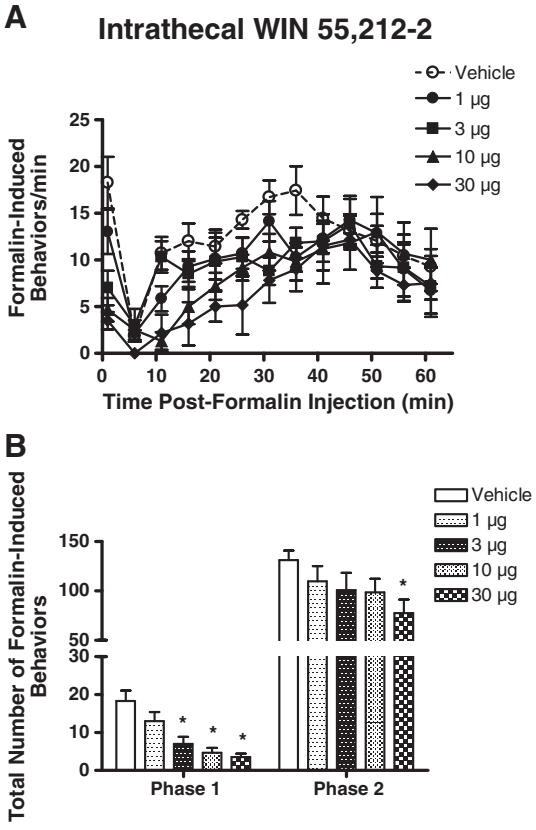

Fig. 2. Effect of intrathecal WIN 55,212-2 on pain-related behavior in the formalin test in rats. A. The horizontal axis is time post-formalin injection (min) and the vertical axis is total number of formalin-induced behaviors counted per minute. Rats were intrathecally (i.t.) injected with 5 μl of a dose (in μg) of WIN 55,212-2 (WIN) or vehicle 10 min prior to hind paw formalin injection. B. Total number of formalin-induced behaviors during phase 1 (0–1 min) and phase 2 (15–61 min). Data are expressed as mean ± S.E.M. n = 7/group. *$p<0.05$ vs. Vehicle.

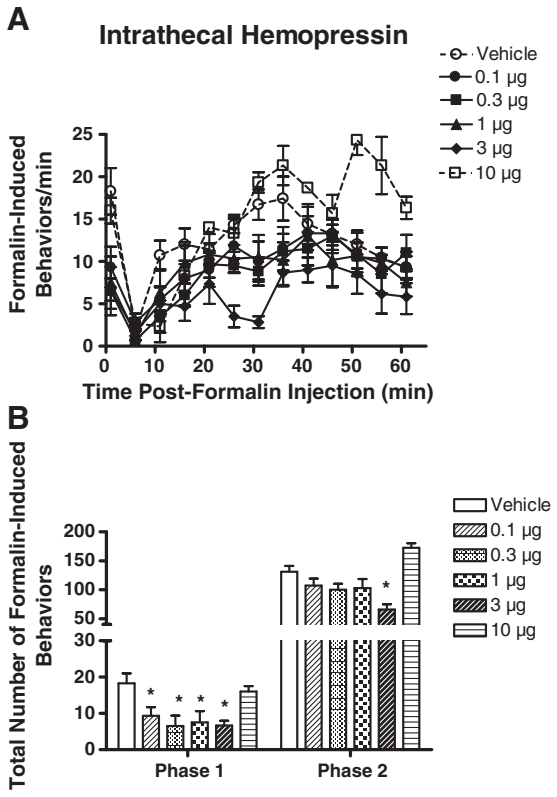

Fig. 3. Effect of intrathecal hemopressin on pain-related behavior in the formalin test in rats. A. The horizontal axis is time post-formalin injection (min) and the vertical axis is total number of formalin-induced behaviors counted per minute. Rats were i.t. injected with 5 μl of a dose (in μg) of hemopressin (HE) or vehicle 10 min prior to hind paw formalin injection. B. Total number of formalin-induced behaviors during phase 1 (0–1 min) and phase 2 (15–61 min). Data are expressed as mean ± S.E.M. n = 6/group. *$p<0.05$ vs. Vehicle.

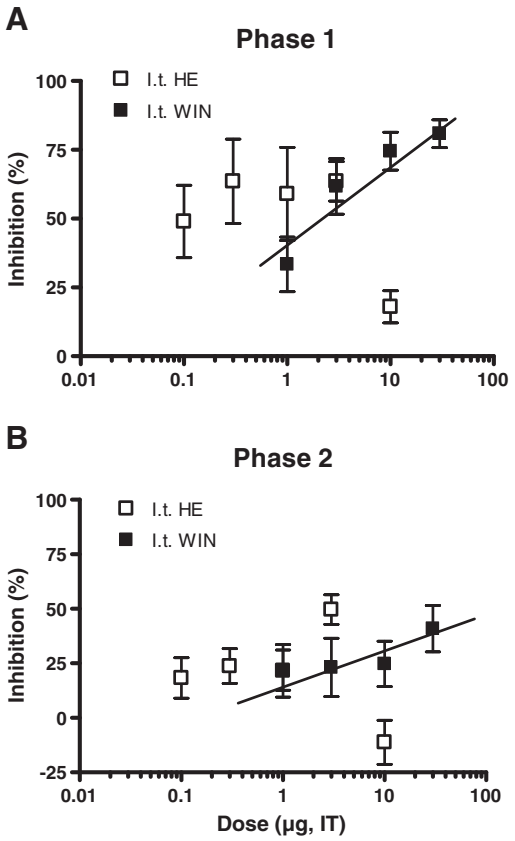

Fig. 4. Best-fit dose–response curves of intrathecal hemopressin and WIN 55,212-2 in the formalin test. The horizontal axis is log-dose (μg) and the vertical axis is percent inhibition (i.t. vehicle treatment = 0% inhibition). The solid lines in (A) and (B) are best-fit curves for WIN 55,212-2 (WIN) A. Increasing i.t. doses of WIN and hemopressin (HE) reduced formalin-evoked pain-related behaviors in phase 1. However, 10 μg HE did not significantly affect formalin-induced behaviors. The A_{50} (95% C.L.) of WIN is 2.2 (1.2–4.2) μg. The estimated A_{50} for HE is 0.6 μg. B. The doses of 30 μg WIN 55,212-2 and 3 μg HE significantly reduced pain-related behaviors in phase 2. The dose of 10 μg HE tended to increase formalin-induced behaviors in phase 2. Data are expressed as mean ± S.E.M.

To determine if supraspinal injection of HE lead to antinociception in the formalin test, 3 μg and 10 μg HE were i.c.v. injected before hind paw formalin injection. Neither dose reduced pain-related behavior (Fig. 5A). On the contrary, phase 2 pain-related behaviors were significantly increased with 3 μg HE ($p<0.05$ vs. vehicle) whereas a trend of elevated behaviors was obtained with 10 μg HE ($p>0.05$ vs. vehicle). By contrast, i.c.v. injection of 10 μg WIN 55,212-2 significantly attenuated total behaviors in both phase 1 and phase 2, 71 ± 10% and 35 ± 10% reversal, respectively ($p<0.05$ vs. vehicle; Fig. 5B).

3.2.1. Effect of intrathecal pretreatment with CB receptor ligands on intrathecal WIN 55,212-2 antinociception in the formalin test

The dose of 1 μg HE was chosen to block the antinociceptive effect of WIN 55,212-2 since this dose itself did not reduce pain-related behaviors in phase 2 of the formalin test. Intrathecal pretreatment with 1 μg HE did not affect the antinociceptive effect of i.t. 30 μg WIN 55,212-2 in phase 2 (Fig. 6A; $p<0.05$, HE/WIN 55,212-2 vs. Vehicle/Vehicle). By contrast, pretreatment with rimonabant blocked the antinociceptive effect of WIN 55,212-2 in both phases 1 and 2 (Fig. 6B; $p>0.05$ rimonabant/WIN 55,212-2 vs. Vehicle/Vehicle).

4. Discussion

The current study compared the antinociceptive effects of the peptide CB1 receptor ligand HE and the subtype nonselective CB receptor agonist WIN 55,212-2 in the hot plate test and the hind paw formalin test following central administration in rats. Whereas neither i.t. HE nor WIN 55,212-2 demonstrated an antinociceptive effect to an acute noxious heat stimulus, both demonstrated significant efficacy in a model of tissue injury-induced sensitization. Injection of WIN 55,212-2 into the lateral ventricle leads to an expected antinociception in both the hot plate and formalin tests. By contrast, i.c.v injection of HE was not antinociceptive in either test. In fact, i.c.v. HE increased pain-related behaviors in the formalin test. To test a potential CB1 receptor antagonist function at the spinal level, HE was i.t. injected prior to i.t. WIN 55,212-2 in the formalin test. Whereas i.t. pretreatment with CB1 receptor antagonist rimonabant blocked the antinociceptive effect of WIN 55,212-2, pretreatment with HE did not. The current preclinical data suggests that HE antinociception may be obtained in an injured but not uninjured state. A CB1 receptor-related mechanism could explain the effect of HE, but given the lack of an antagonistic effect of HE on WIN 55,212-2, other non-CB receptor mediated mechanisms cannot be entirely excluded.

Activation of the CB receptor is a reasonable method of inducing analgesia. Systemic administration of CB receptor agonists leads to significant antinociception in a variety of rat models of tissue injury as well as in uninjured rats (Choong et al., 2007; De Vry et al., 2004; Fox et al., 2001; Hama and Sagen, 2007; Herzberg et al., 1997; Martin et al., 1991; Zhu et al., 2009). Functional CB1 receptors have been identified in CNS areas that modulate nociception (Chin et al., 2008; Herkenham et al., 1991; Horti et al., 2006; Howlett et al., 2004). Based on neuroanatomical studies, dorsal horn CB1 receptors are mainly post-synaptic, on spinal neurons, although a small percentage of CB1 receptors are also of pre-synaptic, primary afferent origin (Agarwal et al., 2007; Farquhar-Smith et al., 2000). Pretreatment with a CB1 receptor antagonist to block either brain or spinal CB1

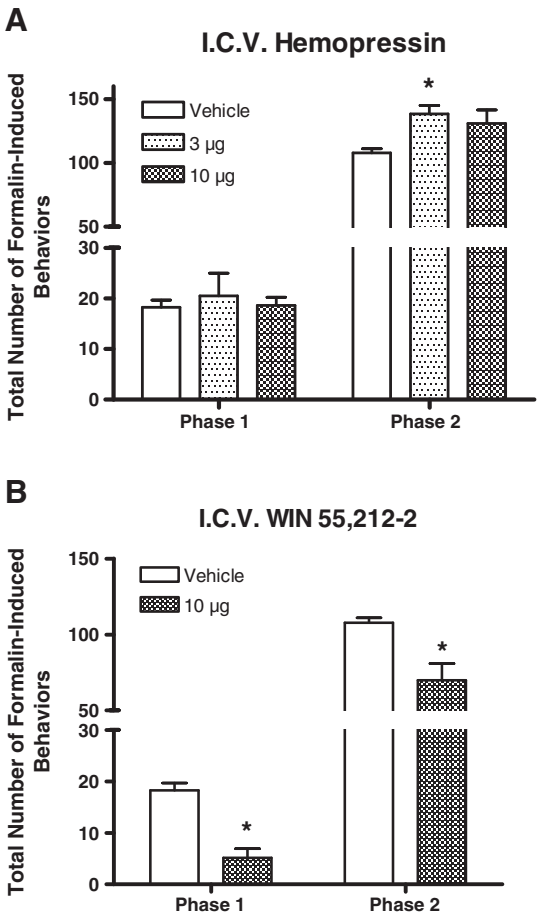

Fig. 5. Effect of intracerebroventricular (i.c.v.) injection of WIN 55,212-2 and hemopressin on pain-related behaviors in the formalin test in rats. Total number of formalin-evoked behaviors during phase 1 (0–1 min) and phase 2 (15–61 min) are shown. Five microliters of either (A) WIN 55,212-2, (B) HE, or vehicle were i.c.v. injected 10 min prior to hind paw formalin injection. Data are expressed as mean ± S.E.M. n = 3–7/group. *$p<0.05$ vs. Vehicle.

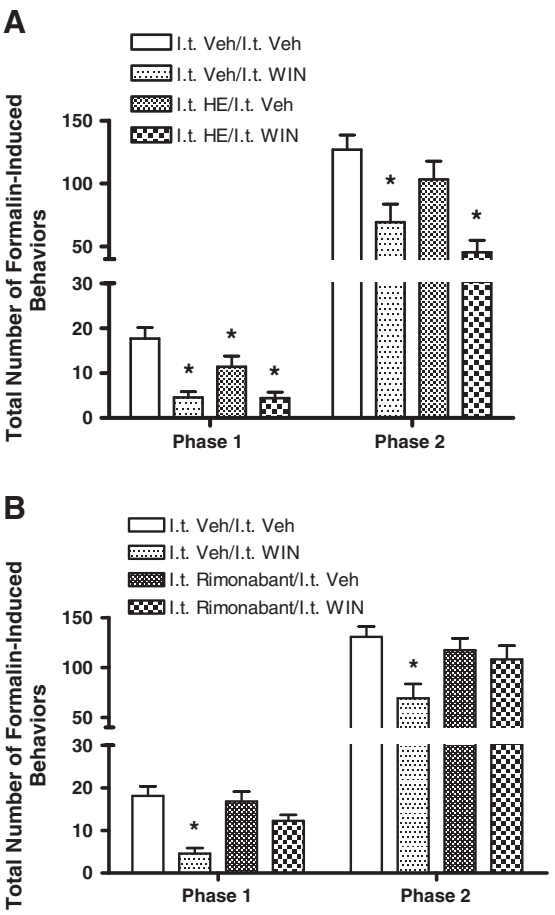

Fig. 6. Effect of intrathecal hemopressin and rimonabant pretreatment on the antinociceptive effect of intrathecal WIN 55,212-2 in the formalin test. A. Rats were i.t. injected with either 1 μg HE or vehicle (Veh) 10 min prior to i.t. injection of 30 μg WIN (or Veh). Ten min following the second i.t. injection, the left hind paw was injected with formalin. B. Rats were i.t. injected with either 30 μg rimonabant or Veh 10 min prior to i.t. injection of 30 μg WIN (or Veh). Ten minutes following the second i.t. injection, the left hind paw was injected with formalin. Data expressed as total number of formalin-induced behaviors in phase 1 (0–1 min) and phase 2 (15–61 min); mean ± S.E.M. n = 7/group. *$p<0.05$ vs. Veh/Veh.

receptors results in a loss of the antinociceptive effect of CB receptor agonists (Choong et al., 2007; Fox et al., 2001; Martin et al., 1999; Welch et al., 1998). Cannabinoid receptor agonists have demonstrated clinical efficacy in various chronic pain states (Iskedjian et al., 2007; Martin-Sanchez et al., 2009; Walker and Huang, 2002).

Accompanying CB-mediated antinociception, however, are characteristic supraspinally mediated side-effects, including catalepsy, hypothermia and hypoactivity (Martin et al., 1991; Pertwee, 1997). In humans, the psychomimetic effects associated with CB receptor activation could offset the beneficial clinical effects. In addition, long-term systemic exposure to CB receptor agonists could lead to dependence and to tolerance to the beneficial effects (De Vry et al., 2004).

One possible way to minimize the side-effects associated with systemic administration of CB receptor agonists is to deliver drugs spinally (Bennett et al., 2000; Pertwee, 2005b). The i.t. space could also be utilized for the implantation of genetically engineered cells that continuously produce analgesic substances (Eaton, 2006; Hentall and Sagen, 2000). Thus, implanting cells in the i.t. space expressing the endogenous peptide HE could be a long-term method of pain relief via continuous engagement of CB receptors. As part of assessing the potential clinical value of spinally delivered HE, activity following acute central delivery of HE in rat pain models was evaluated.

In the current study, the in vivo behavioral effects of HE markedly differed from that of WIN 55,212-2. While efficacy was obtained with i.c.v. injection of WIN 55,212-2 in the hot plate test, no efficacy was observed with i.c.v. HE. A positive association between human postoperative analgesia and antinociceptive efficacy in the hot plate test in rats has been reported for opioid receptor agonists (Yaksh et al., 1999). A similar association between a human pain state and a preclinical acute pain test has yet to be directly demonstrated for CB receptor agonists, but the preclinical and clinical data in total suggests a positive association. The lack of HE antinociception in acute pain tests, noxious heat in the current study and noxious pressure (Heimann et al., 2007), suggests a potential lack of efficacy of HE in human pain states. However, other clinical analgesics, such as non-steroidal anti-inflammatory drugs (NSAID), do not show efficacy in tests of acute nociception such as the hot plate (Malmberg and Yaksh, 1992). Tissue injury may be necessary to "uncover" HE's antinociceptive potential. Therefore, HE was evaluated in the formalin test.

There are two behavioral phases following hind paw formalin injection which are markedly distinct in terms of neurological mechanisms (Coderre et al., 1990; Wheeler-Aceto and Cowan, 1991). The initial "acute" phase is mediated by an intense barrage of peripheral nociceptor activity and the subsequent "chronic" phase is mediated by sustained spinal dorsal horn neuron hyperactivation, driven by the combined effects of changes to dorsal horn synaptic neurochemistry and prolonged nociceptor afferent activity (Dickenson and Sullivan, 1987; Puig and Sorkin, 1996). On an extended time scale, similar changes to dorsal horn neurons have been noted following inflammatory and neuropathic injury; changes to dorsal horn neuron function are believed to underlie

hypersensitivity and increased responsiveness to cutaneous stimulation and spontaneous pain (Millan, 1999). Intrathecal pretreatment with drugs that block dorsal horn changes in inflammatory biochemistry, such as NSAIDs, and block post-synaptic receptor function, such as *N*-methyl-D-aspartate (NMDA) receptor antagonists, prevent the on-set of the physiological and neurochemical changes underlying the chronic phase of the formalin test (Chaplan et al., 1997; Malmberg and Yaksh, 1992). The predominant post-synaptic localization of CB1 receptors indicates that activation of CB1 receptors should attenuate formalin-induced dorsal horn activity and significantly ameliorate pain-related behaviors.

The significant antinociception obtained following i.t. HE injection in the formalin test is consistent with a previous study, albeit in a different injury model (Heimann et al., 2007). The current study generally corresponded to the previous study in terms of HE potency in the acute phase, whereas a higher dose was needed to obtain efficacy in the chronic phase. A commonality between studies that demonstrated antinociceptive efficacy with CB1 receptor antagonist treatment is the use of a pain model with a robust peripheral inflammation. Heimann et al. (2007) hypothesized that peripheral inflammation induces the production of endocannabinoids which increase activity of dorsal horn neurons, leading to cutaneous hypersensitivity (Guasti et al., 2009; Petrosino et al., 2007). A CB1 receptor-mediated production of inflammatory substances, such as cytokines, also contributes to injury-induced hypersensitivity (Costa et al., 2005; Croci et al., 2003). Thus, blocking the pro-nociceptive effects of endocannabinoids and inflammatory processes with a CB1 receptor antagonist at the spinal level should lead to antinociception.

It should be pointed out, however, that i.t. administration of rimonabant in some models of injury-induced pain increases, rather than decreases, pain-related behavior (Martin et al., 1999; Pertwee, 2005a). In other models, including the formalin test, neither hyperalgesic nor antinociceptive effects was obtained following i.t. rimonabant treatment (Khodayar et al., 2006; Sagar et al., 2010). The current study found no significant changes to behaviors following i.t. rimonabant in the formalin test. The various effects observed by blocking CB1 receptors could be entirely dependent on the particular injury. In addition, given the lack of an antinociceptive effect of i.t. rimonabant, the effect of spinal HE in the current study could be non-CB1 mediated.

Intracerebroventricular injection of HE in other pain models has not been previously evaluated (Heimann et al., 2007). A novel finding of the current study was the hyperalgesic effect of i.c.v. HE in the formalin test. The differential effect following i.c.v. and i.t. injection could be due to differences in supraspinal vs. spinal CB1 receptor function or to the recruitment of brain nuclei that project to the spinal dorsal horn and modulate nociceptive neurons (Herkenham et al., 1991; Martin et al., 1995; Welch et al., 1998; Zhu et al., 2009). *N*-methyl-D-aspartate receptors, for example, have shown striking differences in pharmacological properties between supraspinally and spinally expressed receptors (Nakazato et al., 2005). Alternatively, the i.c.v. effect of HE in the formalin test could be due to a non-CB1 receptor-mediated mechanism. Whereas i.c.v. WIN 55,212-2 was antinociceptive in both the formalin test and the hot plate test, i.c.v. HE was not antinociceptive in either. The current study found neither an increase nor a decrease in formalin-induced behaviors following i.c.v. rimonabant and higher doses, used elsewhere, did not lead to hyperalgesia (Lichtman and Martin, 1997). Given the divergent behavioral effects of i.c.v. HE, a non-CB1 receptor-mediated mechanism cannot be ruled out.

Whatever the mechanism, the hyperalgesic effect of i.c.v. HE could in part be mediating the hyperalgesic effect of i.t. 10 μg HE in the formalin test. Significant biological activity of HE persists for at least several hours following i.t. injection, indicating that HE does not appear to be readily degraded (Heimann et al., 2007). Thus, it is possible that HE could have diffused to the brain from the lumbar intrathecal space. Whether hyperalgesia occurs following i.c.v. HE injection in other pain models, such as neuropathic, is not known. Such information will be needed, in addition to the half-life of HE, in order to determined whether i.t. HE treatment should be limited to particular pain states.

One method to address the involvement of the CB1 receptor in the antinociceptive effect of HE is to use mice in which the CB1 receptor has been genetically deleted (Sain et al., 2009). These knockout mice display normal responses to acute noxious and innocuous stimuli. A previous study in CB1 receptor knockout mice confirmed that CB1 receptors mediated the antinociceptive effect of rimonabant (Costa et al., 2005). Thus, a lack of efficacy of i.t. HE in CB1 knockout mice would confirm a CB1 receptor-mediated antinociceptive effect. The persistence of efficacy, however, in knockout mice would indicate the involvement of non-CB1 receptors in HE's effect.

Although HE shares in vitro characteristics of rimonabant, HE did not block the effect of WIN 55,212-2 and HE's behavioral effects were strikingly dissimilar to that of rimonabant, suggesting the possibility of activity at non-CB1 receptors as well as CB1 receptors. Since the ultimate goal is long-term pain management, the effect of HE will need to be evaluated with extended treatment. In fact, the antinociceptive effects of rimonabant in previous studies were revealed following repeated dosing over a seven day period (Costa et al., 2005; Croci and Zarini, 2007). The antinociceptive effect of prolonged rimonabant treatment was sustained with the discontinuation of treatment, indicating a lack of antinociceptive tolerance (Costa et al., 2005). The optimal dosing of HE should also be carefully determined, given the possibility that some of the peptide may diffuse to the brain. With its intrinsic properties, HE appears to be ideal for long-term for pain relief in that it does not appear to induce other CB receptor-mediated behaviors (e.g. catalepsy) and efficacious on injury-induced nociception but sparing normal pain perception.

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