Capsazepine, a Transient Receptor Potential Vanilloid Type 1 (TRPV1) Antagonist, Attenuates Antinociceptive Effect of CB1 Receptor agonist, WIN55,212-2, in the Rat Nucleus Cuneiformis

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Article info:
Received: 10 April 2011
First Revision: 22 April 2011
Accepted: 12 May 2011

Key Words:
Nucleus Cuneiformis,
TRPV1 Receptor,
Cannabinoids,
Tail-Flick Test,
Formalin Test,
Rat

ABSTRACT

Introduction: Nucleus cuneiformis (NCF), as part of descending pain inhibitory system, cooperates with periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) in supraspinal modulation of pain. Cannabinoids have analgesic effects in the PAG, RVM and NCF. The transient receptor potential vanilloid type 1(TRPV1) can be activated by anandamide and WIN55,212-2 as a cannabinoid receptor agonist. The aim of the current study is to investigate the possible interplay between the cannabinoid and vanilloid systems for modulation of pain at the NCF.

Methods: In this study, a cannabinoid receptor agonist, WIN55,212-2 (15 µg/0.3 µl DMSO), and selective TRPV1 receptor antagonist, capsazepine (10, 25, 50 and100 nmol/0.3 µl DMSO), were microinjected bilaterally into the NCF, and tail-flick and formalin tests were used to assess the animal's pain-related behaviors at 5-min intervals for a 60-min period.

Results: Our findings demonstrated that analgesic effect of WIN55,212-2 were dose-dependently attenuated by capsazepine in both tests. In the tail-flick test, capsazepine at both doses of 50 ($P<0.01$) and 100 ($P<0.001$) nmol could significantly prevent the antinociceptive effect of WIN55,212-2 while capsazepine, in formalin test, could decreased its antinociceptive effect at the dose of 50 nmol ($P<0.05$) as well. On the other hand, solely administration of the highest dose of capsazepine in both tests did not alter the pain-related behaviors.

Discussion: It suggests a possible role for TRPV1 receptors in NCF-mediated cannabinoid-induced antinociception.

1. Introduction

The nucleus cuneiformis (NCF) is located just ventrolateral to the periaqueductal gray (PAG) (Gioia & Bianchi, 1987) and it is involved in supraspinal processing of pain (Haghparast & Ahmad-Molaei, 2009; Haghparast, Gheitasi, & Lashgari, 2007; Haghparast, Ordikhani-Seyedlar, & Ziaei, 2008; Haghparast, Soltani-Hekmat, Khani, & Komaki, 2007; Rezvanipour, Milan, & Haghparast, 2006). Several lines of evidence have highlighted the importance of cannabinoids in nociceptive process-

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ing. Exogenous cannabinoids reduce responsiveness to noxious stimuli with a potency and efficacy similar to that of morphine (Bloom, Dewey, Harris, & Brosius, 1977; Jacob, Ramabadran, & Campos-Medeiros, 1981). Cannabinoids have been shown to produce analgesia in neuroanatomical regions subserving transmission and modulation of pain signals, including the PAG, rostral ventrolateral medulla (RVM), basolateral nucleus of amygdala (BLA) (Hasanein, Parviz, Keshavarz, & Javanmardi, 2007; Martin et al., 1999; Martin, Patrick, Coffin, Tsou, & Walker, 1995; Martin, Tsou, & Walker, 1998) and the NCF (Ebrahimzadeh & Haghparast, 2011). Our recent study showed that antinociceptive effects of WIN55,212-2 in the NCF are mediated, at least partly, by CB1 receptors (Ebrahimzadeh & Haghparast, 2011) and it is suggested the non-cannabinoid receptors such as transient receptor potential vanilloid type 1 (TRPV1) receptors may be involved in this phenomenon at the level of NCF.

TRPV1 receptor is activated by heat and capsaicin (Caterina et al., 1997). Other activators of TRPV1 receptors are cannabinoids (Jeske et al., 2006). Electrophysiological and behavioral studies have provided evidence for participation of TRPV1 receptors in the transmission and modulation of nociceptive input (Kelly & Chapman, 2002a,b; Ohkubo, Shibata, & Takahashi, 1993). Mice lacking TRPV1 receptors failed to develop heat hyperalgesia after inflammation (Davis et al., 2000) and in response to microinjection of capsaicin (TRPV1 receptor agonist) into the dorsolateral PAG produces hypoalgesia. The observed effects were absent in capsazepine-pretreated animals, indicating that the TRPV1 receptors may contribute to this antinociception (Palazzo et al., 2002). It has been shown that cannabinoid agonists including WIN55,212-2, anandamide and cannabidiol, activate both CB1 and TRPV1 receptors (Bisogno et al., 2001; Jeske et al., 2006; Pertwee, 2006). The presence of TRPV1 receptors has been recognized in the prefrontal cortex, hippocampus, amygdala, hypothalamus, PAG, locus coeruleus, and cerebellum (Mezey et al., 2000; Roberts, Davis, & Benham, 2004; Sanchez, Krause, & Cortright, 2001; Toth et al., 2005).

Co-expression of TRPV1 and CB1 receptors in several brain regions (Cristino et al., 2006) raises this possibility that cannabinoids could simultaneously act upon both receptors. TRPV1 receptors have a key role in the antinociceptive effect of anandamide at the level of spinal cord (Horvath, Kekesi, Nagy, & Benedek, 2008). In the ventrolateral PAG, cannabinoids modulate pain signals not only through CB1 receptors, but also via TRPV1 receptors (Maione et al., 2006). This study was designed to investigate the possible role of TRPV1 receptors on antinociception induced by exogenous cannabinoid agonist, WIN55,212-2, in the NCF. We examine the effect of bilateral microinjection of (R)-(+) –[2,3-dihydro-5-methyl]-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin 6-yl]-1-naphthalenyl-methanone mesylate (WIN55,212-2), with capsazepine (TRPV1 receptor antagonist), on behavioral manifestations of the animal pain during the tail-flick and formalin tests in rats.

2. Materials & Methods

2.1. Animals

In present study, one-hundred and seventy male albino Wistar rats weighing 250-350g were used. They were housed three per cage in a temperature-controlled room under a 12-h light/dark cycle. All experiments were executed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health Publication No.80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2. Stereotaxic Surgery

Animals were anesthetized with intraperitoneal (i.p.) injection of a mixture of ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg). Experimental animals were prepared with bilaterally guide cannulae implantation into the NCF, at the following stereotaxic coordinates from the atlas of Paxinos and Watson (Paxinos & Watson, 2005): -8.4 mm posterior to bregma; ±1.9 mm lateral; and -6.3 mm ventral from the skull surface. Drugs and vehicle (DMSO) were administered into the NCF through guide cannulae (23-gauge) by lowering a stainless steel injector cannula (30-gauge needle) connected by polyethylene tubing (PE-10) to a 1-µl Hamilton microsyringe. Drug solutions were prepared freshly on the test day and infused in a 0.3 µl volume at the rate of 0.1 µl/15 sec counted on a timer-controlled micrometer and after the completion of the drug infusion, the injector cannula was left in place for a 60 sec extra time and followed by replacement of the obdurator. The following drugs were used: WIN55,212-2, Capsazepine (Tocris Bioscience, Bristol, UK) and DMSO (Sigma-Aldrich, Germany). All drugs were dissolved in DMSO. Formaldehyde, 2.5%, was made from 1 part formalin (~ 36.6%; formalin, Fluka) and 13.64 parts saline.

2.3. Nociceptive Tests

2.3.1. Tail-Flick Test

The nociceptive threshold was measured by the tail-flick apparatus (Harvard, USA). Tail-flick latency (TFL) was measured by exposing the dorsal surface of the rat's tail (3, 5 and 7 cm from the caudal tip of the tail) to radiant heat and recording the onset of moving the tail away from the noxious thermal stimulus. The reaction time between the onset of the heat stimulus and the movement of the tail were determined by an automatic sensor as TFL. The light source was set at 35% of maximum intensity that yields baseline TFL value in the range of 3-4 sec (Haghparast & Ahmad-Molaei, 2009; Haghparast et al., 2008). To avoid tissue damage, the trial was automatically terminated if a response did not occur within 10 sec (cut-off point). The TFLs were measured after the last microinjection into the NCF for a period of 60 min at 5-min intervals. TFLs (sec) are expressed either as raw data or percentage of maximal possible effect (%MPE) which was calculated from the following formula:

$$\%\ \text{MPE} = \frac{\text{Post-drug latency (sec)} - \text{Baseline latency (sec)}}{\text{Cut-off value (sec)} - \text{Baseline latency (sec)}} \times 100$$

To evaluate the sensitivity of animals to nociceptive stimulus, we considered the rat's TFL before drug treatment as a baseline pain threshold.

2.3.2. Formalin Test

Rats were placed in a transparent open Plexiglas chamber (35×35×35 cm) used for observing the animal's behavior during the formalin test. A mirror was positioned at an angle of 45° to permit an unobstructed view of the paw by observer. After the microinjection of either vehicle or the drugs, each rat was given a subcutaneous injection of formalin (2.5%, 50 µl) into the hind paw. Nociception was quantified by assigning weights to the following pain-related behaviors (Coderre, Fundytus, McKenna, Dalal, & Melzack, 1993; Dubuisson & Dennis, 1978; Hasanein et al., 2007; Manning & Franklin, 1998). Rats were observed for a 60-min period following formalin injection and the time spent in each type of behavior was recorded in 5-min blocks. The four behavioral categories are as follows: 0, the position and posture of the injected hind paw is indistinguishable from the other hind paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category by the category weight, summing these products and dividing by the total time (300 sec) for each 5-min block of time.

$$\text{Nociceptive score} = (t_0 \times 0) + (t_1 \times 1) + (t_2 \times 2) + (t_3 \times 3)/(t_0 + t_1 + t_2 + t_3)$$

An ordinal scale (Coderre et al., 1993) of nociceptive scores was generated with a range of 0-3.

2.4. Experimental Protocols

In this study, by using tail-flick and formalin tests, we examined the effect of antagonizing TRPV1 receptors on cannabinoid-induced analgesia in the NCF. Therefore, there are two sets of experiments in the tail-flick and formalin tests: (1) microinjection of capsazepine+WIN55,212-2 into the NCF. In this set of experiment, WIN55,212-2 (15µg/0.3 µl DMSO per side) was bilaterally microinjected into the NCF following microinjection of the different doses of capsazepine (10, 25, 50 and 100 nmol/0.3 µl DMSO per side) and (2) microinjection of capsazepine alone into the NCF. In this set of experiment, DMSO as a vehicle of WIN55,212-2 (0.3 µl per side) was bilaterally microinjected into the NCF following microinjection of the highest dose of capsazepine in both tests. In control respective group, animals received DMSO alone into the same region, bilaterally.

2.5. Statistical Analysis

The results obtained are expressed as mean ± SEM. The mean TFLs in all groups were subjected to one-way and/or two-way ANOVA followed by protected Dunnett/Newman-Keuls and/or Bonferroni tests for multiple comparisons as needed. In order to evaluate the nociceptive responses, area under the curve (AUC) for each group of animals in tail-flick or formalin test was calculated as raw TFLs or pain scores × time by linear trapezoidal method (Heinzen & Pollack, 2004; Wegner et al., 2008), respectively, and a single value for each control/ experimental group was used in statistical analysis. The calculated AUC values in all groups were subjected to one-way ANOVA followed by protected Dunnett's or Newman-Keuls multiple comparison tests. P-values less than 0.05 were considered to be statistically significant.

2.6. Histology

After the experiments, rats were anesthetized with ketamine and xylazine and were transcardially perfused with 0.9% saline, followed by 10% buffered formalin. The brains were cut coronally in 50 µm sections stained with Cresyl violet through the cannula placements. Injection sites were histologically verified with the atlas of Paxinos and Watson (Paxinos & Watson, 2005). Eleven rats were excluded due to cannula misplacement (Supplementary figure 1).

3. Results

In control groups, in the tail-flick test, two-way ANOVA for repeated measures over time followed by Bonferroni's test for TFLs revealed that there were no significant differences in TFLs at any time intervals among the intact (n=6), sham-operated (n=6) and vehicle groups [treatment main effect: $F(2,195)=0.8796$, $P=0.4166$; time main effect: $F(12,195)=0.8288$, $P=0.6207$; treatment×time interaction: $F(24,195)=0.7528$, $P=0.7915$]. In another set of experiments, formalin test, two-way ANOVA for repeated measures over time followed by Bonferroni's test for obtained pain score values [treatment main effect: $F(2,180)=1.332$, $P=0.1682$; time main effect: $F(11,180)=5.222$, $P=0.0062$; treatment×time interaction effect: $F(22,180)=0.4949$, $P=0.9725$] revealed that there were no significant differences in formalin-induced pain behaviors among the intact, sham-operated and vehicle groups. All animals were compared to respective DMSO group and its pain score value results were considered as baseline in all 5-min blocks in both tests.

3.1. Effects of intra-NCF administration of capsazepine alone and in combination with WIN55,212-2 on TFLs in tail-flick test

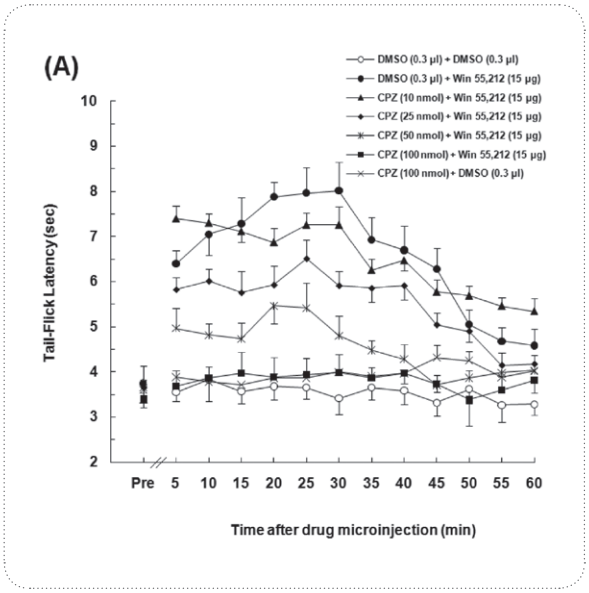
To evaluate the effect of antagonizing TRPV1 receptors within the NCF on antinociceptive response of WIN55,212-2 in tail-flick test as a model of acute pain, different doses of capsazepine (10, 25, 50 and 100 nmol/0.3 µl DMSO per side), a selective TRPV1 receptor antagonist, were microinjected into the NCF just before intra-NCF administration of WIN55,212-2 (15 µg/side). TFLs were measured at 5-min intervals during 60 min period. Fig.1A showed that intra-NCF administration of capsazepine could significantly attenuate the antinociceptive responses of WIN55,212-2 in a dose-dependent manner [treatment main effect: $F(6,481)=121.8$, $P<0.0001$; time main effect: $F(12,481)=13.99$, $P<0.0001$; treatment×time interaction: $F(72,481)=2.515$, $P<0.0001$]. Notably, the antinociceptive response of WIN55,212-2 was completely suppressed by the highest dose of capsazepine. In contrast, administration of capsazepine (100 nmol) alone could not affect the baseline. One-way ANOVA followed by Newman-Keuls multiple comparison test also showed that there were significant differences in mean AUC values among the control and experimental groups [$F(6,43)=50.5$, $P<0.0001$; Fig.1B]. Different doses of capsazepine dose-dependently decreased the AUC values as compared to mean AUC value in WIN-control animals that received solely WIN55,212-2 (15 µg/side) into the NCF.

3.2. Effects of intra-NCF administration of capsazepine alone and in combination with WIN55,212-2 on time-course of formalin-induced persistent pain behaviors

In the formalin test as a model of inflammatory persistent pain, to evaluate the role of TRPV1 receptors within the NCF in antinociceptive response of WIN55,212-2, different doses of capsazepine (10, 25 and 50 nmol/0.3 µl DMSO per side) were microinjected into the NCF just before intra-NCF administration of WIN55,212-2 (15 µg/side) and then the weighted pain scores were recorded at 5-min blocks during a 60 min period. Fig. 2A showed that intra-NCF administration of capsazepine could significantly prevent the antinociceptive responses of WIN55,212-2 in a dose-dependent manner [treatment main effect: $F(5,360)=37.76$, $P<0.0001$; time main effect: $F(11,360)=5.915$, $P<0.0001$; treatment×time interaction: $F(55,360)=3.612$, $P=0.0426$]. On the other hand, there were no significant differences in formalin pain scores in all time set intervals between control (DMSO) group and animals that received only the highest doses of capsazepine (50 nmol/side) into the NCF. Mean AUC calculated results in Fig. 2B revealed that different doses of capsazepine dose-dependently increased the AUC values as compared to that in WIN-control animals that received solely WIN55,212-2 (15 µg/side) into the NCF. Increase in AUC values in this figure are considered as a nociceptive index. One-way ANOVA followed by Newman-Keuls multiple comparison test also showed that the antinociceptive response of WIN55,212-2 was completely suppressed by 50 nmol dose of capsazepine [$F(5,35)=5.37$, $P=0.0012$; Fig.2B].

4. Discussion

The major findings in this study were (a) antinociceptive effects of intra-NCF administration of WIN55,212-2 on pain-related behaviors in the tail-flick and formalin



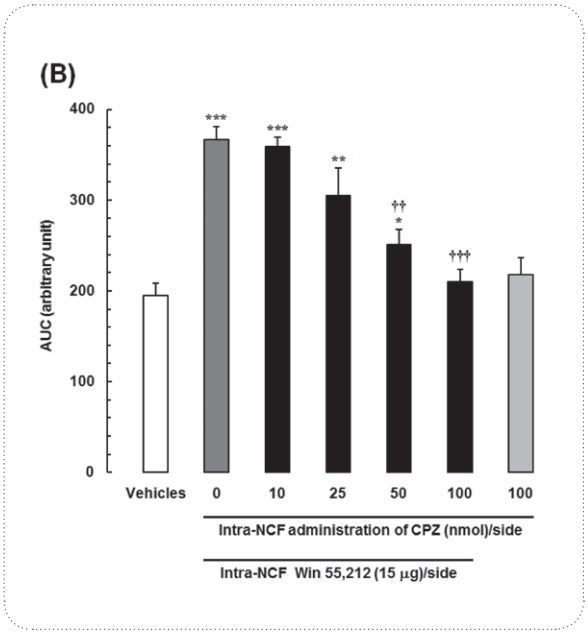


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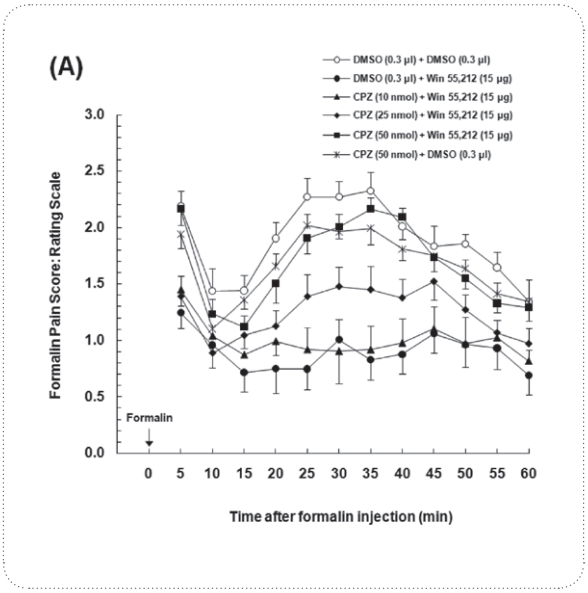
Figure 1. (A) Time-dependent curves representing the effects of administration of different doses of capsazepine, a selective TRPV1 receptor antagonist alone, and in combination with WIN55,212-2 (15 µg/side) into the nucleus cuneiformis (NCF) in the tail flick test. (B) The mean area under the curves (AUCs) was obtained from the time-response curves shown in A. In vehicle group, animals received DMSO (0.3 µl/side) into the NCF bilaterally. Data are represented as mean ± SEM for 6 rats.

* P<0.05; ** P<0.01; *** P<0.001 compared to DMSO control group
†† P<0.01; ††† P<0.001 compared to DMSO+Win55212,2 (Win control) group



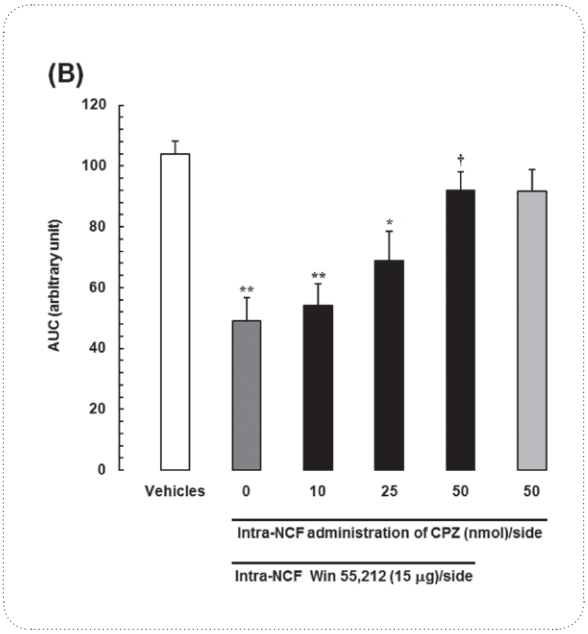


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Figure 2. (A) Time-course of formalin-induced pain behaviors representing the effects of bilateral intra-nucleus cuneiformis (NCF) administration of different doses of capsazepine, a cannabinoid TRPV1 receptor antagonist, vehicle (DMSO; 0.3 µl/side) and WIN55,212-2 (15 µg/side) on pain-related behaviors in the formalin test. (B) Area under the curves (AUCs) was obtained from the time-course responses shown in A. Data are represented as mean ± SEM for 6 rats.

* P<0.05; ** P<0.01 compared to Vehicles group
† P<0.05 compared to DMSO+Win55212,2 (Win control) group

tests were significantly reduced in a dose-dependent manner with a prior microinjection of capsazepine (TRPV1 receptor antagonist), and (b) intra-NCF administration of capsazepine alone did not have any effect on pain-related behaviors in both tests.

Several lines of evidence indicate the existence of a cannabinoid pain modulatory system in different brain regions including the PAG (Hohmann et al., 2005; Martin et al., 1999; Palazzo et al., 2001; Suplita, Richard, Farthing, Gutierrez, & Hohmann, 2005; Walker, Huang, Strangman & Tsou, 1999), RVM (Martin et al., 1998; Meng & Johansen, 2004; Meng, Manning, Martin, & Fields, 1998; Monhemius, Azami, Green, & Roberts, 2001), BLA (Hasanein et al., 2007) and NCF (Ebrahimzadeh & Haghparast, 2011). Our results, indicating the antinociceptive effects of intra-NCF administration of WIN55,212-2 in the tail-flick and formalin tests, are consistent with the results of investigations in these regions (Martin et al., 1995, 1998; Meng et al., 1998). The observed antinociceptive effect of WIN55,212-2 in the NCF was significantly antagonized by high doses of capsazepine in both tests. Attenuation of WIN55,212-2 effects by capsazepine has been reported at the level of spinal cord (Horvath et al., 2008). Pistis et al. (2004) also showed an antagonizing effect of capsazepine in an electrophysiological study in the amygdale (Pistis et al., 2004). Our results demonstrated that intra-NCF administration of capsazepine alone has no effect on the expression of pain-related behaviors in tail-flick and formalin tests. This is along with the results of study by Perkins and Campbell (1992) about the neutral effect of capsazepine on nociception, when administered alone (Perkins & Campbell, 1992). In contrary, it has been shown that capsazepine and several other TRPV1 receptor antagonists affect noxious thermosensation (Gunthorpe et al., 2007; Tang et al., 2007). The present findings provide an evidence for a correlation between antagonizing the TRPV1 receptors and attenuation of WIN55,212-2-mediated antinociception in the NCF. Based on this correlation, a possible role could be suggested for TRPV1 receptors in NCF-mediated cannabinoid-induced analgesia. To demonstrate the presence and specific cell localization of cannabinoid and TRPV1 receptors in the NCF, other experiments like immunohistochemical and bimolecular analyses could be possible useful approaches. Also, for better elucidating, the possible role of cannabinoids and vanilloids in the NCF-mediate analgesia, single unit extracellular recording experiments can be designed to measure the cell activity in the NCF in response to chemical (formalin-induced persistent pain) or thermal stimuli before and after pharmacological manipulation of cannabinoid and vanilloid receptors.

Acknowledgments

The authors would like to thank Mr. Mahmoudreza Ramin for editing our manuscript. This work was supported by the grant (No. 88-301-A) from Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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