# Glutamate Receptors in Nucleus Accumbens Can Modulate Canabinoid-Induced Antinociception in Rat's Basolateral Amygdala

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## ABSTRACT

**Introduction:** It has been shown that administration of WIN55,212-2, a cannabinoid receptor agonist, into the basolateral amygdala (BLA), dose-dependently increases the thermal latency to withdrawal in the tail-flick test and decreases pain related behaviors in both phases of the formalin test. Recent human and animal imaging data suggest that the nucleus accumbens (NAc) is an important neural substrate of pain modulation. Because the NAc also receives abundant glutamatergic fibers from the BLA which converge with hippocampal fibers on the same NAc neurons, it is reasonable to ask whether AMPA/kainate and NMDA receptor antagonists may also include in the amygdala-accumbens pathway in pain modulation.

**Methods:** In the present study, we examined the role of NMDA and AMPA/kainate receptors within the NAc in antinociception induced by intra-BLA injection of the cannabinoid receptor agonist WIN55,212-2 in rats. Seventy two adult male albino Wistar rats weighing 230-280 g were implanted with two separate cannulae into the BLA and the NAc. Also, animals received intra-accumbal infusions of either NMDA receptor antagonist, AP5 (0.5, 2.5 and 5 µg/0.5 µl saline) or AMPA/kainate receptor antagonist, CNQX (0.1, 0.5 and 2.5 µg/0.5 µl DMSO) 2 min before microinjection of WIN55,212-2 into the BLA (15 µg/rat).

**Results:** Antinociceptive effects of WIN55,212-2 were measured in the formalin test (50 µl injection of formalin 2.5% subcutaneously into the hindpaw) and pain-related behaviors were monitored for 60 min. Results showed that intra-accumbal AP5 and CNQX dose-dependently prevented antinociception induced by intra-BLA administration of WIN55,212-2 in time set intervals. Additionally, intra-accumbal AP5 administration of both AP5 (5 µg/0.5 µl saline) and CNQX (2.5 µg/0.5 µl DMSO), alone, could not significantly change the pain scores in the rats.

**Discussion:** It seems that glutamate receptors located in the NAc, partially mediate the antinociceptive responses of cannabinoid within the BLA in persistent inflammatory model of pain.

## 1. Introduction

The amygdala has been divided into several nuclei based on cytoarchitectural, histochemical, connectional, and functional criteria (Swanson & Petrovich, 1998). The basolateral amygdala (BLA) complex (lateral, basolateral, and basomedial nuclei) and the central nucleus are associated with affective conditioning and responding (Cardinal, Parkinson, Hall, & Everitt, 2002). The BLA receives projections from areas including the medial prefrontal cortex (mPFC) (Cassell, Chittick, Siegel, & Wright, 1989), sensory association cortex (Mascagni, McDonald, & Coleman, 1993), and thalamus (Turner & Herkenham, 1991). Inputs from the BLA to central nuclei and onto autonomic and neuroendocrine centers constitute an important pathway in the induction of different kinds of emotional, autonomic

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and neuroendocrine responses (Pitkanen, Savander, & LeDoux, 1997).

It has been repeatedly shown that cannabinoid signaling plays an important role in controlling nociception (Ebrahimzadeh & Haghparast, 2011; Parvishan, Taslimi, Ebrahimzadeh, & Haghparast, 2011; Roche, O'Connor, Diskin, & Finn, 2007). Direct evidence for the involvement of supraspinal cannabinoid receptors in the modulation of pain has been obtained from a number of studies employing intracerebral microinjection of cannabinoids or endocannabinoid system modulators in animal models of acute, inflammatory or neuropathic pain (Rea, Roche, & Finn, 2007). Further studies demonstrated that intra cerebroventricular injection of non-selective cannabinoid receptor agonists suppressed nociception in the rat tail-flick test, and these antinociceptive effects were reversed by the CB1 receptor antagonist such as rimonabant (Lichtman, Cook, & Martin, 1996; Martin, Tsou, & Walker, 1998). Martin et al. (1999) demonstrated that the cannabinoid receptor agonist, WIN55,212-2, shows antinociceptive effects in the tail-flick test when injected into a number of rat brain regions including subnuclei of the amygdala, thalamus, periaqueductal gray (PAG) and rostroventral medulla (RVM) (Martin, et al., 1999). Additional evidence supporting a role for the amygdala as an important site mediating cannabinoid-induced antinociception comes from the work demonstrating that bilateral lesions to the amygdala abolish the antinociceptive effects of systemically administered WIN55,212-2 in the tail-flick test in rhesus monkeys (Manning, Merin, Meng, & Amaral, 2001).

On the other hand, the NAc receives excitatory glutamatergic afferents from limbic regions such as the BLA, prefrontal cortex, and hippocampus (Brog, Salyapongse, Deutch, & Zahm, 1993; Pennartz, Groenewegen, & Lopes da Silva, 1994), and in many instances, inputs from these anatomically and functionally distinct regions converge on the same medium spiny neurons (Floresco, Blaha, Yang, & Phillips, 2001; Mulder, Hodenpijl, & Lopes da Silva, 1998). Extensive experimental and clinical evidence suggests a presynaptic location of cannabinoid receptors on GABAergic and glutamatergic neurons in brain areas associated with pain modulation (Rea, et al., 2007). Excessive glutamate receptor activation plays a major role in spinally mediated nociception (Nishiyama, Gyermek, Lee, Kawasaki-Yatsugi, & Yamaguchi, 1999). AMPA/kainate receptors mediate fast excitatory transmission involving both innocuous and acute nociceptive input, whereas NMDA receptors are implicated specifically in nociceptive responses, particularly those induced by intense, prolonged stimulation sufficient to produce the hyperalgesic state underlying neuropathic pain (Dougherty, Palecek, Paleckova, Sorkin, & Willis, 1992; Nishiyama, et al., 1999). It has been suggested that the blockade of AMPA/kainate receptors located in the spinal cord appears to be involved in enhancing the inhibition of tail-flick responses induced by stimulation of spinal mu-, delta- and kappa-opioid receptors (Suh, Song, Huh, & Kim, 2000). Therefore, in the current study, the following experiments were designed and we tried to examine whether glutamatergic receptors in the NAc mediate the antinociceptive responses of cannabinoids within the BLA in the formalin test as a rat model of persistent inflammatory pain.

## 2. Methods

### 2.1. Animals

Seventy two male albino Wistar rats weighing 250-350g were used as subjects. They were housed three per cage in a temperature and light controlled room under a 12-h light/dark cycle with free access to chow and tap water. 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 Neuroscience Research Center, Shahid Beheshti University of Medical Sciences.

### 2.2. Surgical Preparation

The rats were anesthetized with intraperitoneal (i.p.) injection of ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) and cannulae were stereotaxically (Stoelting, stereotaxic apparatus, USA) implanted in the BLA and/or NAc. The coordinates for these regions were determined by the rat brain atlas (Paxinos & Watson, 2005) as AP=2.8 mm caudal to bregma, Lat=±4.6 mm lateral to midline, DV=8.7 mm ventral from the skull surface for BLA (guide cannula was 2 mm above the appropriate injection place) and for the NAc the coordinates were: AP=1.2 mm rostral to bregma, Lat=±1.6 mm lateral to midline and DV=7.8 mm ventral from the skull surface (guide cannula was 1 mm above the appropriate injection place). The guide cannulae were secured in place using two stainless steel screws anchored to the skull and dental acrylic cement. After the cement was completely dried and hardened, two stainless steel stylets were used to occlude the guide cannulae during recovery period. Animals were individually housed and allowed to recover for 5-7 days before testing.

## 2.3. Drug Administration

Microinjections were performed by lowering stainless steel injector cannulae (30-gauge needle) with a length of 1 mm longer than the guide cannulae into the NAc and 2 mm longer than the guide cannulae into the BLA. The injector cannulae were connected to a 1-µl Hamilton syringe by polyethylene tubing (PE-20). In the present study, the following drugs were used: WIN55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3,-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) as a cannabinoid receptor agonist (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany), AP5 (DL-2-Amino-5-phos-phonopentanoic acid) as a NMDA receptor antagonist (Tocris Bioscience, Bristol, UK) was dissolved in saline and CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) as an AMPA/kainate receptor antagonist (Tocris Bioscience, Bristol, UK) was dissolved in DMSO as a vehicle. Control animals received either saline and/or DMSO. All of microinjections were performed unilaterally in this study. Formalin test was conducted at the same times during the day.

## 2.4. Formalin Test

Rats were placed in the transparent open Plexiglas chamber (35×35×35 cm) with a mirror angled at 45° to be used for observing the animal's behavior during the formalin test. A mirror was positioned at an angle of 45° to permit unhindered observation of the animal's paw under the chamber. After the microinjection of either vehicle or the drugs, each rat was given a formalin subcutaneous injection (2.5%, 50 µl) into the hind paw. Subjects in this experiment were observed for 60 min following formalin injection. Nociception was quantified by assigning weights to the following pain related behaviors (Haghparast & Ahmad-Molaei, 2009; Haghparast, Ghalandari-Shamami, & Hassanpour-Ezatti, 2012) and the time spent in each type of behavior was recorded in 5 min blocks for 60 min test period. The four behavioral categories are as follows: 0, the position and posture of the injected hind paw was indistinguishable from the another hind paw; 1, the injected paw had little or no weight placed on it; 2, the injected paw was elevated and was not in contact with any surface; 3, the injected paw was licked, bitten or shaken. Afterward, 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.

$$\text{Nociceptive score} = (t_0\times0) + (t_1\times1) + (t_2\times2) + (t_3\times3)/ (t_0 + t_1 + t_2 + t_3)$$

By utilizing this method, an ordinal scale (Haghparast, Naderi, Khani, Lashgari, & Motamedi, 2010; Haghparast, et al., 2012) of nociceptive scores was generated with a range of 0-3.

## 2.5. Experimental Protocols

### 2.5.1. Effect of intra-accumbal NMDA receptor antagonist AP5 on antinociception induced by administration of WIN55,212-2 into the basolateral amygdala

To evaluate the effect of NMDA receptor antagonist on antinociceptive responses of cannabinoid receptor agonist, animals unilaterally received AP5 (0.5, 2.5 and 5µg/0.5µl saline; n=6 in each group) in the NAc and 2 min later, the highest dose of WIN55,212-2 (15µg/rat) was microinjected into the BLA. Additionally, in another group of animals, the highest effective dose of AP5 (5µg/0.5µl saline; n=6) was administered alone in the NAc, while animals had received DMSO instead of WIN55,212-2 in the BLA. In the vehicles group (n=6 in each group), saline was microinjected into the NAc and 2 min later animals received DMSO in the BLA.

### 2.5.2. Effect of administration of AMPA/kainate receptor antagonist CNQX into the NAc on antinociception induced by intra-BLA cannabinoid receptor agonist

In order to examine the possible role of AMPA/kainate receptor in the NAc in cannabinoid receptor agonist-induced antinociception in the BLA, CNQX was unilaterally microinjected into the NAc at various doses (0.1, 0.5 and 2.5µg/0.5µl DMSO; n=6in each group), just 2 min before administration of WIN55,212-2 in the BLA. In another set of experiment, rats only received the highest effective dose of CNQX (2.5µg/0.5µl DMSO; n=6) in the NAc before microinjection of DMSO (0.3µl/rat) instead of cannabinoid receptor agonist, WIN55,212-2, in the BLA. In the vehicles group (n=6), rats received DMSO in both the NAc and BLA nuclei.

## 2.6. Statistical Analysis

The obtained results are expressed as mean ± SEM (standard error of mean). In order to evaluate the nociceptive responses, area under the curves (AUCs) was calculated as raw pain scores × time by linear trapezoidal method and a single value was used in statistical analysis. The calculated AUC and pain score values in

all groups were subjected to one-way and/or two-way ANOVA followed by protected Tukey's or Bonferroni's test for multiple comparisons, respectively. P-values less than 0.05 were considered to be statistically significant.

### 2.7. Histology

After completion of the experiments, rats were deeply anesthetized with ketamine and xylazine and were transcardially perfused with 0.9% saline and 10% formaldehyde solution prior to sectioning. Then, rats were sacrificed and their brains were removed. The neuroanatomical locations of cannulae tips were confirmed using Paxinos and Watson rat brain atlas (2005). The data reported here are only from animals in which the placements of cannulae sites were histologically verified.

## 3. Results

Obtained results for formalin pain score revealed that there are no significant differences in formalin pain scores at any time intervals among the intact (n=5), sham-operated (n=5) and vehicles (Saline/DMSO delivered into the NAc/BLA in a volume of 0.5/0.3 µl per side; n=5) groups. Hence, all experimental animals were compared to respective Saline/DMSO group as a control and their results were considered as baseline in all time set intervals. Newman-Keuls multiple comparison test also showed that there are no significant differences in the mean calculated AUCs for formalin pain scores [F(2,14)=0.3982, P=0.6801] among the intact, sham-operated and vehicle treated groups. In the next experiment, we used the same protocol of our recent study (Ghalandari-Shamami, Hassanpour-Ezatti, & Haghparast, 2011) and the dose-response effects of intra-BLA administration of WIN55,212-2 (5, 10 and 15 µg/0.3 µl DMSO per rat), a cannabinoid agonist, on formalin pain score, 60 min after microinjection in formalin test. Newman-Keuls multiple comparison tests showed that there are significant differences in the mean calculated AUCs, for pain scores, among the experimental and vehicle (DMSO) groups. AUCs calculated for pain scores in formalin test showed that the most effective dose of WIN55,212-2 is 15 µg/rat. Henceforth, this dose was chosen for the next experiments.

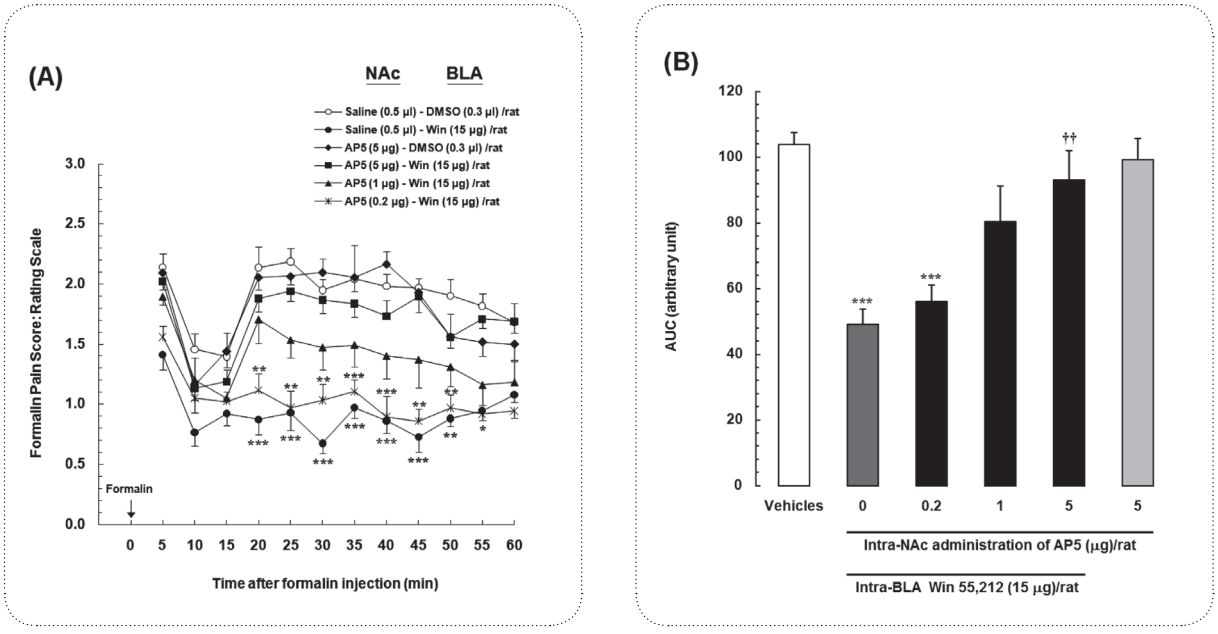


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**Figure 1.** Effects of administration of different doses of AP5, a NMDA receptor antagonist, into the nucleus accumbens (NAc) on antinociception induced by WIN55,212-2 microinjected into the basolateral amygdala (BLA) in the formalin test. (A) The average of pain scores (pain behaviors) in 60-min period after formalin injection; and (B) area under the curves (AUCs) calculated for formalin pain scores shown in A. In vehicles group, animals received saline (0.5 µl) into the NAc and DMSO (0.3 µl) into the BLA, unilaterally. In WIN55,212-2 control group, animals received solely WIN55,212-2 (15 µg/0.3µl DMSO) into the BLA. Data are represented as mean ± SEM for 6 rats.

* P<0.05; ** P<0.01; *** P<0.001 compared to Saline/DMSO control (vehicles) group

†† P<0.01 compared to WIN55,212-2 control group

### 3.1. Effects of intra-accumbal administration of AP5, a NMDA receptor antagonist, on antinociception induced by intra-BLA cannabinoid receptor agonist in formalin tests

In the first set of experiments, we examined the dose response effects of different doses of AP5 (0.2, 1 and 5µg/0.5µl saline per rat), a selective NMDA receptor antagonist, microinjected into the NAc, on antinociception induced by intra-BLA administration of WIN55,212-2 (15µg/rat; the most effective dose) during 60 min period. Figure 1A showed that intra-accumbal administration of different doses of AP5 (0.2, 1 and 5µg/0.5µl saline per rat), significantly decreased the antinociceptive effect of cannabinoid receptor agonist, WIN55,212-2, microinjected into the BLA [treatment main effect: $F(5,360)=59.68$, $P<0.0001$; time main effect $F(11, 360)=7.774$, $P<0.0001$; treatment × time interaction $F(55,360)=0.8893$, $P=0.6963$]. One-way ANOVA followed by Newman-Keuls multiple comparison test showed that there were significant differences in AUC calculated values of pain scores in this set of experiments [$F(5,35)=8.742$, $P<0.0001$Fig. 1B]. Although AP5 (5µg/rat) could significantly ($P<0.01$) decrease the most antinociceptive effect of intra-BLA WIN55,212-2, administration of maximal dose of AP5 (5µg/rat) alone into the NAc could not affect the pain scores at time set intervals and/or AUC calculated value in comparison with vehicles group.

### 3.2. Effects of intra-accumbal administration of CNQX, a AMPA/kainite receptor antagonist, on antinociception induced by WIN55,212-2 microinjected into the basolateral amygdala

In another set of experiments, we examined the dose response effects of different doses of CNQX (0.1, 0.5 and 2.5µg/0.5µl DMSO per rat), a selective AMPA/Kainate receptor antagonist, microinjected into the NAc, on antinociception induced by intra-BLA administration of WIN55,212-2 (15 µg/rat) during 60 min period in persistent inflammatory animal models of pain. Intra-accumbal administration of different doses of CNQX (0.5 and 2.5µg/0.5µl DMSO per rat), significantly decreased the antinociceptive effect of cannabinoid receptor agonist, WIN55,212-2, microinjected into the BLA [treatment main effect: $F(5,360)=79.17$, $P<0.0001$; time main effect $F(11, 360)=7.623$, $P<0.0001$; treatment ×

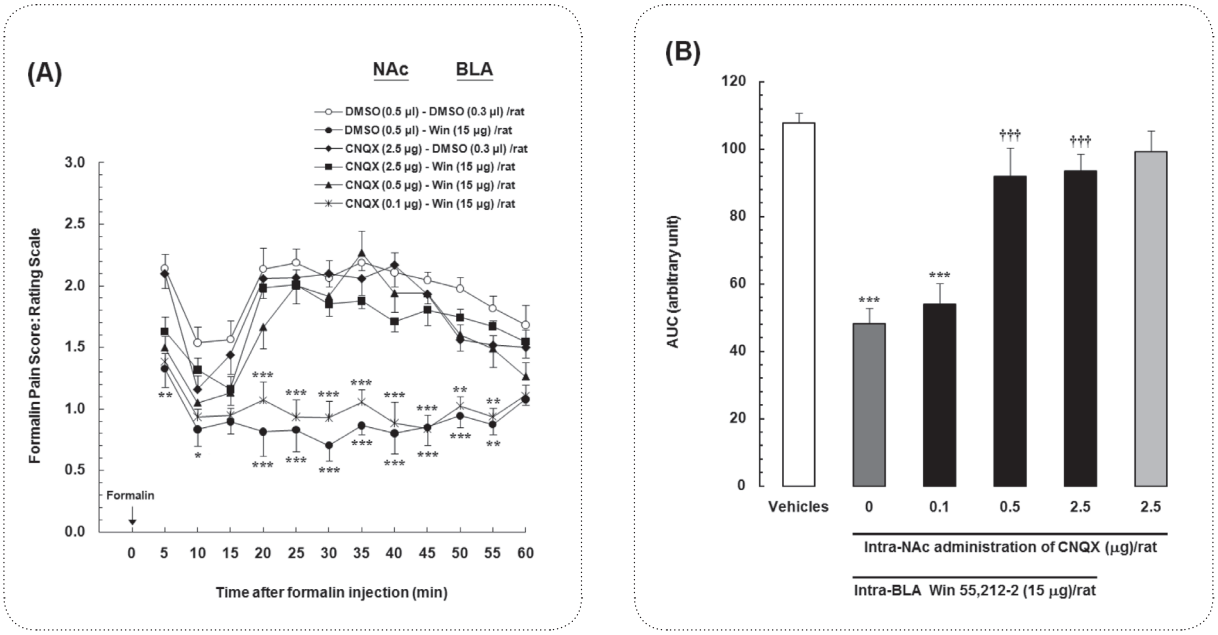


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**Figure 2.** Effects of intra-accumbal (NAc) administration of different doses of CNQX, a AMPA/kainate receptor antagonist, on antinociception induced by WIN55,212-2 microinjected into the basolateral amygdala (BLA) in the formalin test. (A)The average of pain scores (pain behaviors) in 60-min period after formalin injection; and (B) area under the curves (AUCs) calculated for formalin pain scores shown in A. In vehicles group, animals received DMSO into the NAc(0.5 µl) and the BLA(0.3 µl), unilaterally. In WIN55,212-2 control group, animals received solely WIN55,212-2 (15 µg/0.3µl DMSO) into the BLA. Data are represented as mean ± SEM for 6 rats.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to DMSO control (vehicles) group

††† $P<0.001$ compared to WIN55,212-2 control group

time interaction F(55,360)=1.43, P=0.0607; Fig. 2A]. Moreover, as shown in Fig. 2B, one-way ANOVA followed by Newman-Keuls multiple comparison test revealed that there are significant differences in AUC calculated values for pain scores in this set of experiments [F(5,35)=18.6, P<0.0001]. Although different doses of CNQX (0.5 and 2.5µg/0.5µl DMSO per rat) could significantly decrease the most antinociceptive effect of intra-BLA WIN55,212-2 (15 µg/rat), administration of maximal dose of CNQX (5µg/rat) alone into the NAc could not affect the pain scores at time set intervals and/or AUC calculated value in comparison with vehicles group.

## 4. Discussion

The purpose of this study was to evaluate involvement of the glutamate receptors within the NAc in antinociceptive responses induced by intra-BLA administration of cannabinoid receptor agonist in rats. The major findings were: (a) AMPA/kainite receptor antagonism in the NAc prevented the antinociceptive responses of intra-BLA administration of cannabinoid receptor agonist formalin tests (b) microinjection of NMDA receptor antagonist in the NAc could inhibit WIN55,212-induced analgesia in formalin test (c) Intra-NAc administration of NMDA or AMPA/kainite receptors antagonist alone, could not significantly change the pain scores in formalin test.

We showed that microinjection of AMPA/kainate receptor antagonist into the NAc can prevent the antinociceptive effects induced by microinjection of cannabinoid receptor agonist into the BLA. This finding is consistent with other studies showing that AMPA/kainite receptors mediate fast neurotransmission in the central nervous system (Hartmann, et al., 2004). Evidence in support of a therapeutic potential for AMPA/kainite receptor antagonists in chronic pain states comes from studies implicating a role for AMPA/kainate receptors in nociceptive signaling, as well as the actions of selective compounds in animal models of persistent inflammatory pain. Ionotophoretic and systemic application of 2,3 benzodiazepine selective AMPA/kainate antagonists has been shown to suppress firing of wide dynamic range neurons in the dorsal horn in response to both noxious and innocuous stimuli, suggesting that AMPA/kainate receptors at the level of the spinal cord are involved in both normal sensory and nociceptive transmission (Budai & Larson, 1994). Several pharmacological studies using a variety of animal models of experimental pain have confirmed a role for AMPA/kainite receptors in persistent pain states. For example, the development of spinal sensitization and secondary hyperalgesia in incisional and first degree burn models can be prevented by pretreatment with intrathecal AMPA/kainate receptor antagonists NBQX or CNQX (Bleakman, Alt, & Nisenbaum, 2006). On the other hand, our previous study showed that AMPA/kainate receptors located in the NAc, in part, could not mediate the antinociceptive responses of cannabinoid within the BLA in tail-flick model of pain (Ghalandari-Shamami, et al., 2011). But in the present study, it seems that AMPA/kainite receptors within the NAc, in normal situation, are important in pain modulation in model of persistent inflammatory pain.

In another part of the study, our results indicated that NMDA receptor antagonism in NAc, prevented the antinociceptive responses of intra-BLA administration of cannabinoid receptor agonist in formalin test. Neuroimaging studies have correlated signal responses in the amygdala with pain behaviors in animals and NMDA-receptor mediated synaptic plasticity in the amygdala appears to involve phosphorylation of GLUN1 subunits (Bird, et al., 2005; Bleakman, et al., 2006). In a previous study, it was shown that NMDA receptors are involved in morphine-induced analgesia (Jacquet, 1988). Several studies indicated that spinal NMDA receptors play a pivotal role in the development of tonic pain. Previous studies have reported that NMDA receptor antagonists, such as AP5, AP7, can induce antinociception in the tail flick test in rodents (Lutfy, Cai, Woodward, & Weber, 1997). Additionally, administration of AP5, a NMDA receptor antagonist alone, into the NAc cannot significantly change formalin pain score. Lutffy et al. (1997) showed that intrathecal administration of non-NMDA receptor antagonists induces antinociception in Swiss Webster mice in the tail flick test, an animal model of phasic pain, whereas selective NMDA receptor antagonists are ineffective. They suggested that activation of the non-NMDA receptors are necessary for transmission of phasic pain, whereas, activation of NMDA and/or non-NMDA receptors may be involved in mediation of tonic pain (Lutfy, et al., 1997). Haghparast et al. (2007) suggested that NMDA but not non-NMDA receptors are involved in the antinociception produced by morphine in the nucleus cuneiformis (CnF). The non-NMDA receptors in this area may have a facilitatory effect on nociceptive transmission (Haghparast, Gheitasi, & Lashgari, 2007). Also, they suggest that morphine related antinociceptive effect elicited from the CnF is mediated, in part, by NMDA receptor at the level of the NRM, whereas kainite/AMPA receptor has a net inhibitory influence at the same pathway (Haghparast, Soltani-Hekmat, Khani, & Komaki, 2007).

In conclusion, our data suggests that administration of NMDA receptor, and non-NMDA receptor antagonists in the NAc partially mediate the antinociceptive responses of cannabinoid within the BLA in persistent inflammatory model of pain. Indeed, glutamate receptor in the NAc can modulate the cannabinoid induced antinociception in the BLA. It seems that glutamatergic projections from the BLA to the NAc may be necessary for potent analgesic effects of cannabinoid. However, further pharmacological and electrophysiological investigations are needed to elucidate the hypothesis of the actual role of glutamate receptors in the NAc; and these mechanisms are involved in modulating cannabinoid induced antinociception in animal models of pain. Also, we need a deep investigation to provide more information about these receptors, such as injecting a retrograde tracer (fluorogold; FG) in the NAc and look for FG-positive cell bodies in the BLA, and to perform an immunohistochemical experiment to determine whether the FG-positive cell bodies express CB1 receptors or not.

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