

## **Different Effects of Opioid and Cannabinoid Receptor Agonists on C-fiber-Induced ERK Activation in Dorsal Horn Neurons in Normal and Spinal Nerve-Ligated Rats**

Yasuhiko Kawasaki, Tatsuro Kohno, and Ru-Rong Ji

Pain Research Center, Department of Anesthesiology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115 (YK, RRJ)

<sup>2</sup>Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan (TK)

Running title: Spinal ERK activation and opioids and cannabinoids

Number of text pages: 22

Number of tables: 0

Number of figures: 6

Number of references: 40

Number of words in the Abstract: 249

Number of words in the Introduction: 500

Number of words in the Discussion: 1456

Correspondence:

Ru-Rong Ji, Department of Anesthesiology, Brigham and Women's Hospital

75 Francis Street, Medical Research Building, Room 604, Boston, MA 02115

Tel: (617) 732-8852; Fax: (617) 730-2801

Email: rrji@zeus.bwh.harvard.edu

**ABBREVIATIONS:**

ACEA, arachidonyl - 2'-chloroethylamide

ANOVA, analysis of variance

BDNF, brain-derived neurotrophic factor

CB1, cannabinoid receptor subtype-1

CB2, cannabinoid receptor subtype-2

CCK, cholecystokinin

DAMGO, [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin

DRG, dorsal root ganglion

ERK, extracellular signal-regulated kinase

GIRK, G protein-coupled inwardly rectifying potassium channel

IR, immunoreactive

MAPK, mitogen-activated protein kinase

MOR,  $\mu$  opioid receptor

SNL, spinal nerve ligation

TRPV1, transient receptor potential subtype-1

## ABSTRACT

Nerve injury results in neuropathic pain, a debilitating pain condition. While cannabinoids are consistently shown to attenuate neuropathic pain, the efficacy of opioids is highly controversial. Molecular mechanisms underlying analgesic effects of opioids and cannabinoids are not fully understood. We have shown that the signaling molecule ERK (extracellular signal-regulated kinase) are activated by C-fiber stimulation in dorsal horn neurons and contributes to pain sensitization. In this study, we examined whether opioids and cannabinoids can affect C-fiber-induced ERK phosphorylation (pERK) in dorsal horn neurons in spinal cord slices from normal and spinal nerve ligated rats. In normal control spinal slices, capsaicin-induced a drastic pERK expression in superficial dorsal horn neurons, which was suppressed by morphine (10  $\mu$ M), the selective  $\mu$ -opioid receptor agonist [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin (DAMGO, 1  $\mu$ M), and the selective CB1 receptor agonist arachidonyl - 2'-chloroethylamide (ACEA, 5  $\mu$ M). One week after spinal nerve ligation when neuropathic pain is fully developed, capsaicin induced less pERK expression in the injured L5-spinal segment. This pERK induction was not suppressed by morphine (10  $\mu$ M) and DAMGO (1  $\mu$ M), but enhanced by high concentration of DAMGO (5  $\mu$ M). In contrast, ACEA (10  $\mu$ M) was still very effective in inhibiting capsaicin-induced pERK expression. In the adjacent L4 spinal segment, both DAMGO and ACEA significantly suppressed pERK induction by capsaicin. These results indicate that after nerve injury opioids lose their capability to suppress C-fiber-induced spinal neuron activation in the injured L5 but not in the intact L4 spinal segment, while cannabinoids still maintain their efficacy.

## Introduction

Injury to the nervous system causes neuropathic pain, which is resistant to conventional treatment, e.g. non-steroid anti-inflammatory drugs (Woolf and Mannion, 1999; Ji and Strichartz, 2004). Increasing evidence indicates that cannabinoids are effective in alleviating neuropathic pain via spinal, supraspinal, and peripheral mechanisms (Zelster et al., 1991; Ibrahim et al., 2003; Lim et al., 2003; Cravatt and Lichtman, 2004). In contrast, the efficacy of opioid in treating neuropathic pain is controversial. Neuropathic pain has traditionally been considered opioids-resistant, to intrathecal opioids in particular (Ossipov et al., 1995; Mao et al., 1995; Lee et al., 1995). However, some recent clinical (Eisenberg et al., 2005; Raja and Haythornthwaite, 2005) and animal studies (Zhao et al., 2004; Zhang et al., 2005) have shown that opioids [especially  $\mu$  opioid receptor (MOR) agonists] can be effective in treating neuropathic pain. These conflicting data may result from differences in the type of nerve injury, route of drug administration, potency and receptor selectivity of the particular opioid agonist, and the methods of measuring neuropathic pain. Opioids are believed to inhibit synaptic transmission in dorsal horn spinal cord neurons via both presynaptic and postsynaptic mechanisms. Presynaptically, opioids reduce transmitter release by blocking N-type calcium channels (Glaum et al., 1994; Kohno et al., 2005). Postsynaptically, opioids open G protein-coupled inwardly rectifying potassium (GIRK) channels, hyperpolarizing the membrane (Yushimura et al., 1983; Kohno et al., 2005). However, molecular mechanisms underlying analgesic effects of opioid and cannabinoid are not fully understood. It is not clear whether spinal neurons have different sensitivity to cannabinoids and opioids after nerve injury.

The extracellular signal-regulated protein kinase (ERK) is the best-studied member of the MAPK (mitogen-activated protein kinase) family and plays a critical role in intracellular signal transduction, neural plasticity, and inflammatory responses (Ji and Woolf, 2001). Our previous studies have shown that noxious stimulation (e.g. C-fiber activation) can induce ERK phosphorylation (activation) in dorsal horn neurons, and that inhibition of this phosphorylation by MEK (MAPK kinase) inhibitors suppresses tissue injury-induced pain hypersensitivity (Ji et al., 1999; Kawasaki et al., 2004). ERK activation by inflammation is

also involved in regulating gene transcription and maintaining persistent inflammatory pain (Ji et al., 2002).

Primary afferent nociceptor terminals release several neurotransmitters and neuromodulators in the spinal cord following noxious stimulation. These include the excitatory amino acid glutamate, the neuropeptide substance P, and the brain-derived neurotrophic factor (BDNF). Activation of the corresponding NMDA and metabotropic glutamate receptors, NK-1 receptors, and TrkB receptors in postsynaptic dorsal horn neurons has been implicated not only in the induction of pain sensitization but also in the activation of ERK (Ji and Woolf, 2001; Karim et al., 2001; Lever et al., 2003; Kawasaki et al., 2004). Therefore, ERK activation could be used as a marker of dorsal horn neuron activation following noxious stimulation. Are analgesic effects of opioids and cannabinoids associated with the inactivation of ERK? In the present study, we have investigated the effects of cannabinoids and opioids on capsaicin-induced pERK expression in dorsal horn neurons in normal and nerve ligated neuropathic pain rats.

## **Materials and Methods**

### **Animals and reagents**

Male Sprague-Dawley rats (150-200g) were used under Harvard Medical School Animal Care institutional guidelines. The animal room was artificially illuminated from 7:00 am to 7:00 pm. The rats were anesthetized with sodium pentobarbital (40-50 mg/kg, i.p). To produce a spinal nerve ligation (SNL), the L5 transverse process was removed to expose the L4 and L5 spinal nerves. The L5 spinal nerve was then isolated and tightly ligated with 6-0 silk thread (Kim and Chung, 1992). Capsaicin and DAMGO were purchased from Sigma (St. Louis, MO, USA). ACEA was purchased from Tocris Cookson (Ellisville, MO, USA).

### **Spinal cord slice preparation**

Rat spinal cord slices were prepared as previously described (Ji et al., 1999; Kohno et al., 2003; Kawasaki et al., 2004). Briefly, the lumbar spinal cord was removed and immersed in

cold Krebs's solution, and a 700- $\mu\text{m}$ -thick transverse slice from L4 or L5 spinal segment was cut on a vibrating microslicer. The slice was perfused with Krebs's solution saturated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at  $36 \pm 1^\circ\text{C}$  for more than 3 hours. All the drugs were applied 10 min before capsaicin stimulation and were present during capsaicin stimulation (3  $\mu\text{M}$  for 5 min). Ten minutes after the capsaicin stimulation, the slices were fixed in 4% paraformaldehyde for 60 min, replaced with 20% sucrose overnight, and cut in a cryostat at 15  $\mu\text{m}$ . Because the surfaces of the slices are likely to be damaged during the preparation, we trimmed the slices (100  $\mu\text{m}$  each side) in a cryostat and collected 20-25 serial sections (15  $\mu\text{m}$  thick) only from the middle of the slices. Six to eight non-adjacent sections of these (every third one) were picked for analysis. These sections were then processed for immunofluorescence.

### **Immunohistochemistry**

Spinal sections were processed for immunohistochemistry using the immunofluorescence (Jin et al., 2003; Kawasaki et al., 2004). Briefly, spinal sections were blocked with 2% goat serum in 0.3% Triton for 1 hr at room temperature, and incubated overnight at  $4^\circ\text{C}$  with anti-pERK primary antibody (anti-rabbit, 1:300, Cell Signaling). The sections were then incubated with Cy3-conjugated secondary antibody (anti-rabbit, 1:300, Jackson Immunolab) for 90 min at room temperature following primary antibody incubation. Immunostained slides will be examined under a Nikon fluorescence microscope. All the images will be captured with a high resolution CCD Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA)

### **Quantification and Statistics**

Six to eight non-adjacent sections from the lumbar spinal cord (L4-L5) segments were selected, and the numbers of immunoreactive (IR) neurons for pERK in the laminae I-II (Molander et al., 1984) were counted in each section, as previously described (Kawasaki et al., 2004). The numbers of labeled neurons from 6-8 sections were averaged for each animal and 4-6 rats were included in each group. The data were represented as Mean  $\pm$  SEM. The % inhibition of pERK expression by capsaicin after drug treatment was determined by the formula:  $[100 - 100 \times (\text{number of pERK-IR neurons after drug treatment} - \text{number of basal})]$

pERK-IR neurons) / (number of pERK-IR neurons after capsaicin stimulation alone - number of basal pERK-IR neurons)]. For the calculation of % inhibition, the basal pERK expression was subtracted, because it is not changed after drug treatment (also see Kawasaki et al., 2004). The difference between different groups were compared using ANOVA followed by Fisher's PLSD, and the criterion for statistical significance was  $P < 0.05$ .

## Results

### Effects of opioid and cannabinoid receptor agonists on ERK activation in the normal spinal cord

We used a rat spinal cord slice preparation (700  $\mu\text{m}$ ) in the present study. This preparation offers a reliable condition to study pERK expression (Ji et al., 1999; Lever et al., 2003; Kawasaki et al., 2004). Also multiple slices can be prepared from each spinal cord segment and drugs can be applied in a known condition in vitro. The slices were perfused for more than 3 hours before stimulation to reduce possible pERK background caused by slice preparation (Ji et al., 1999; Kawasaki et al., 2004). There were very few pERK-immunoreactive (IR) neurons in control non-stimulated spinal cord slices (Fig 1a; Fig 2).

To activate C-fibers, we chose capsaicin rather than electrical stimulation, because (a) electrical stimulation at C-fiber intensity will also activate A $\beta$ - and A $\delta$ -fibers, and (b) capsaicin stimulation is much easier to apply. Since the capsaicin receptor TRPV1 (transient receptor potential subtype-1) is only expressed in primary afferent terminals in the superficial dorsal horn, bath application of capsaicin to spinal slices can induce the release of neurotransmitters and neuromodulators (e.g. glutamate, substance P, and BDNF) from TRPV1-expressing primary afferents to activate their postsynaptic receptors, leading to ERK activation in dorsal horn neurons (Kawasaki et al., 2004). A brief exposure of capsaicin (3  $\mu\text{M}$ , 5 min) to spinal slice induced a marked pERK increase in the whole superficial dorsal horn (laminae I-II, Fig 1b, Fig 2). To identify cell types exhibiting pERK expression, we performed double immunofluorescence for pERK and NeuN, a neuronal marker. Almost all pERK-labeled cells were also positive for

NeuN, indicating that pERK is induced in dorsal horn neurons after capsaicin stimulation (Fig 1e). There was no significant difference of pERK expression between L4 and L5 spinal slices: the number of pERK-positive neurons following capsaicin in the L4 and L5 slices was  $22.4 \pm 0.8$  and  $21.9 \pm 1.0$  (n=4), respectively.

Capsaicin-induced pERK increase was significantly suppressed by non-specific MOR agonist morphine (10  $\mu$ M, 56% inhibition,  $P < 0.01$ ) and by the specific MOR agonist DAMGO (78% and 74% inhibition at 1 and 5  $\mu$ M,  $P < 0.01$ , Fig 1c, Fig 2). The effects of DAMGO were mediated by opioid receptors, because the opioid receptor antagonist naloxone (10  $\mu$ M) abolished the DAMGO's effect: the number of pERK-positive neurons after treatment of naloxone (10  $\mu$ M) together with DAMGO (5  $\mu$ M) and capsaicin was  $23.6 \pm 2.5$  (n=4,  $P > 0.05$ , compared to capsaicin). However, a high dose of DAMGO (20  $\mu$ M) did not reduce pERK induction (Fig 2). A selective CB1 agonist ACEA also inhibited capsaicin-induced pERK expression (41% and 55% inhibition at 5 and 10  $\mu$ M, respectively,  $P < 0.01$ , Fig 1d, Fig 2). Neither DAMGO (5 and 20  $\mu$ M, 30 min incubation) nor ACEA (10  $\mu$ M, 30 min incubation) changed the basal expression of pERK (Fig 2). These results indicate that both opioid and cannabinoid receptor agonists, at optimal concentrations are effective in suppressing C-fiber-induced ERK activation in dorsal horn neurons.

### **ERK activation in the spinal cord by capsaicin after spinal nerve ligation**

Ligation of the L5 spinal nerve (SNL) is known to produce a rapid (<1 d) and persistent (>1 month) neuropathic pain, characterized by mechanical allodynia (nociceptive response to normally innocuous low intensity mechanical stimulus) and heat hyperalgesia (increased response to noxious heat stimulus) (Kim et al., 1992). Since nerve injury may change the sensitivity of dorsal horn neurons to cannabinoids and opioids, we further examined the regulation of pERK expression in dorsal horn neurons in the injured L5 segment and adjacent non-injured L4 spinal segment at one week after SNL, when neuropathic pain was fully developed (Kim et al., 1992; Jin et al., 2003). The basal pERK level in the laminae I-II of spinal slices was not significantly different from that of control slices from naïve rats (naïve,  $3.1 \pm 0.1$ ; after SNL: L4,  $3.0 \pm 0.2$ ; L5,  $2.9 \pm 0.1$ ). However, pERK induction by capsaicin in the L5 spinal cord was markedly decreased; the number of pERK-IR neurons



decreased from  $22.4 \pm 1.0$  in naive slices (L4/L5) to  $12.4 \pm 0.5$  ( $P < 0.01$ ) in the L5 slices of injured rats (Figs 3-5). This decrease is likely to be caused by a loss of TRPV1 receptors in primary afferents after nerve injury. pERK-IR neurons after SNL were still localized in the superficial (laminae I-II) dorsal horn (L5) in response to capsaicin stimulation (Fig 3). After SNL, capsaicin-induced pERK expression in the L4 spinal slices was only slightly decreased ( $P < 0.05$ ) compared to naïve slices, pERK induction in the L4 slices was significantly higher ( $P < 0.01$ ) than that in the L5 slices (Fig 4).

### **Effects of opioid and cannabinoid agonists on ERK activation in the spinal cord of nerve injured rats**

Although opioid receptor agonists DAMGO and morphine could inhibit capsaicin-evoked pERK induction in naïve slices (Fig 2), they were not able to suppress the pERK expression in the L5 spinal slices after SNL (Fig 3, 5). In contrast, high dose of DAMGO (5  $\mu\text{M}$ ) could even potentiate capsaicin-induced ERK activation in the L5 spinal cord (28% increase,  $P < 0.01$ , Fig 3). On the other hand, cannabinoid CB1 agonist ACEA was still very effective in suppressing pERK induction by capsaicin (50% and 72% inhibition at 5  $\mu\text{M}$  and 10  $\mu\text{M}$ , respectively,  $P < 0.01$ , Fig 5). The basal pERK expression in the L5 slices ( $3.1 \pm 0.1$ ) was not significantly altered after short exposure (30 min) of DAMGO ( $2.7 \pm 0.1$ , 1  $\mu\text{M}$ ), morphine ( $2.9 \pm 0.1$ , 10  $\mu\text{M}$ ) or ACEA ( $2.9 \pm 0.3$ , 10  $\mu\text{M}$ ).

However, in the L4 spinal slices of nerve-injured rats, capsaicin-induced ERK activation was significantly diminished by DAMGO (1 and 5  $\mu\text{M}$ ,  $P < 0.01$ ), morphine (10  $\mu\text{M}$ ,  $P < 0.01$ ) and ACEA (5 and 10  $\mu\text{M}$ ,  $P < 0.01$ ) (Fig 6).

### **Discussion**

Opioids have been shown to induce ERK activation in several conditions. For example, acute morphine treatment (systemic for 30 min) is shown to induce ERK activation in the anterior cingulate and locus ceruleus (Eitan et al., 2003). Furthermore, modulation of ERK activation by acute morphine is brain-region specific; ERK activation is decreased in the nucleus accumbens and central amygdala (Eitan et al., 2003). Acute cannabinoid treatment

was also shown to induce ERK activation in hippocampal neurons (Derkinderen et al., 2003). However, we did not see significant change in basal pERK expression following a short exposure of opioids and cannabinoids in the spinal cord, supporting the notion that ERK activation by opioids and cannabinoids is tissue-specific. However, it is likely that pERK might be induced in the spinal cord after chronic treatment of opioids and cannabinoids.

In the normal condition, we found both opioids and cannabinoids can suppress C-fiber induced ERK activation. Consistently, previous studies have shown that both opioids and cannabinoids can inhibit noxious stimulation-induced c-Fos expression in the spinal cord (Presley et al., 1990; Tsou et al., 1996). c-Fos expression is a downstream event of ERK activation, since ERK inhibition is shown to suppress c-Fos expression following noxious stimulation (Kawasaki et al., 2004). ERK activation is not only a marker for spinal neuron activation, but also an important contributor to spinal neuron sensitization (Ji et al., 1999; Hu and Gereau, 2003; Kawasaki et al., 2004). Therefore, inactivation of spinal ERK could be a molecular mechanism underlying opioid- and cannabinoid-mediated analgesic effect. It remains to be investigated how opioids and cannabinoids suppress C-fiber stimulation-evoked pERK expression. It is likely that opioids and cannabinoids could regulate ERK phosphorylation via both presynaptic (e.g. reducing neurotransmitter release) and postsynaptic mechanisms (e.g. inhibiting the activity of adenylate cyclase). We have shown that PKA is essential for capsaicin-induced pERK expression (Kawasaki et al., 2004). Therefore, opioids and cannabinoids could suppress pERK expression by inhibiting the activity of adenylate cyclase and subsequent activation of PKA. Whereas in conditions of morphine tolerance, cAMP levels in dorsal horn neurons are increased, leading to opposite regulation of pERK.

Opioid receptors ( $\mu$ ,  $\delta$ ,  $\kappa$  subtype) are normally present in small size (C- and A $\delta$ -fiber) nociceptive dorsal root ganglion (DRG) neurons and their central terminals in the superficial dorsal horn. MOR is also expressed in lamina II dorsal horn neurons (Ji et al., 1995). Nerve injury induces a downregulation of MOR both in DRG neurons and in the spinal cord after nerve injury (Degroat et al., 1997; Porreca et al., 1998; Zhang et al., 1998). Recently, we

have shown that downregulation of MOR is restricted to the injured DRG and spinal segment (Kohno et al., 2005). Nerve injury also induces an upregulation, in DRG neurons of the peptide cholecystinin (CCK) and its receptor CCK<sub>B</sub>, which have been shown to antagonize opioid actions (Wiesenfeld-Hallin et al., 2002). The downregulation of opioid receptors and upregulation of the CCK system were thought to be responsible for the decreased efficacy of opioids after neuropathy (Xu et al., 1993; Wiesenfeld-Hallin et al., 2002). Moreover, the efficacy of opioid suppression of A $\delta$  fiber-induced pre- and post-synaptic electrical responses in the injured spinal segment is also reduced (Kohno et al., 2005). A loss of MOR agonist activity after nerve injury may reflect decreased transcription, translation and trafficking of the receptor to the membrane, as well as an uncoupling of the receptor from G proteins. However, Porreca et al. have shown that nerve injury only reduces the expression level of MOR in the injured spinal segment, but does not change the density or affinity of MOR and morphine's potency in activating G-proteins (Porreca et al., 1998). In contrast, peripheral inflammation increases MOR expression in DRG neurons, in support of increased analgesic sensitivity to opioids (Ji et al., 1995; Shaqura et al., 2004).

In agreement with a loss of MOR in the injured spinal segment, our results showed that DAMGO and morphine have lost their ability to inhibit C-fiber-induced ERK activation in the L5-spinal cord after SNL, suggesting that MOR-mediated intracellular signaling is also impaired in the injured spinal cord. It has been shown that morphine tolerance (a neuronal adaptation to long-term morphine exposure or high dose morphine) develops more rapidly after nerve injury. Opioid could even produce hyperalgesia after the tolerance (Mao et al., 1995; Ossipov et al., 2004). Interestingly, our data has shown that after nerve injury, high dose of opioid can further potentiate C-fiber-induced ERK activation instead of inhibiting ERK activation. Therefore, opioid could both reduce and enhance pERK expression under different conditions, producing both antinociceptive and pronociceptive effects. ERK activation may underlie both antinociceptive and pronociceptive effects of opioid depending on the doses and duration (acute vs chronic) of opioid treatment and animal conditions (naïve vs nerve injured).

In contrast to a diminished opioid efficacy in the L5 segment in response to C-fiber stimulation, CB1 agonist ACEA was fully capable of suppressing C-fiber-induced ERK activation after SNL. CB1 is strongly expressed in the superficial spinal cord, including primary afferent terminals and local neurons (Farquhar-Smith et al., 2000). Moreover, CB1 level is increased in the spinal cord after nerve injury (Lim et al., 2003). It remains to be investigated whether CB2 might also contribute to ERK activation in dorsal horn neurons. However, CB2 is typically expressed in the peripheral non-neural tissue and has been shown to contribute to the development of neuropathic pain via a peripheral mechanism (Ibrahim et al., 2003). CB2 activation produces antinociception by stimulating peripheral release of endogenous opioids (Ibrahim et al., 2005). Recent evidence indicates that CB2 receptor might be induced in spinal microglia after nerve injury (Zhang et al., 2005). Since the psychoactive effects of cannabinoids are mediated by CB1, development of CB2 agonists for pain relief could avoid devastating side effects of CB1 agonists. Recently, we found that pERK is also induced in spinal microglia at early stage (first several days) of nerve injury, which is important for the development of neuropathic pain (Zhuang et al., 2005). It is likely that spinal CB2 agonists may reduce neuropathic pain by suppressing MAPK (p38 and ERK) activation in spinal microglia (Jin et al., 2003; Ji and Strichartz, 2004). However, in spinal cord slices prepared from rats with 1-week nerve ligation, we did not see significant ERK activation in spinal microglia in the superficial spinal cord. This could be due to (a) loss of pERK staining during slice preparation, and (b) decline of microglial pERK induction after one week.

A great advantage to use the spinal nerve ligation model is that the injured and non-injured fibers are clearly separated in the different DRGs (L5 vs L4) and spinal segments (L5 vs L4) in this model. Spontaneous activity, which is believed to play an important role in the generation of neuropathic pain, develops in both the injured and the adjacent intact axons as well as in their somata. A loss of MOR activity on injured primary afferent central terminals and the second order neurons they innervate may minimize the opioid reduction of spontaneous pain mediated by ectopic input from axotomized afferents. On the other hand, our data showed that DAMGO remains its potency to suppress C-fiber-induced pERK in the intact L4 spinal segment after SNL. Thus, retention of MOR activity in nearby intact

afferents will enable an opioid-mediated reduction of stimulus-evoked and spontaneous pain carried by intact nociceptor afferents. It is worthwhile to mention that after SNL, pain signal from the tested hindpaw should be transmitted via intact fibers, since injured axons have lost their connections to the peripheral targets.

In summary, the efficacy of opioids in treating neuropathic pain is a highly debated issue. Recent animal and clinical studies indicate that the potency of opioid remains unchanged at least in some neuropathic pain conditions. However, molecular and cellular mechanisms of opioid actions are only partially known. We have shown that phosphorylation of the signaling molecule ERK could be a useful tool to study neuronal sensitivity to opioid treatment in both normal and nerve injured conditions. Inactivation or activation of spinal ERK is likely to be a molecular mechanism of opioid-induced analgesia or hyperalgesia. Our results support a diminished efficacy of opioid in the injured spinal segment, in response to C-fiber-induced neuronal activation after spinal nerve ligation. Therefore, opioids may reduce sensitivity in those patients whose pain is generated mainly from injured nociceptor discharge. However, opioid may still be able to suppress neuropathic pain via acting on intact primary afferents, or via supraspinal mechanisms. Since cannabinoid's efficacy in suppressing C-fiber-induced pERK expression fully remains in the injured spinal segments after nerve ligation, our results support an undiminished potency of cannabinoid in attenuating neuropathic pain. Our data also suggest that there might be different regulatory mechanisms of opioids and cannabinoids for neuropathic pain.

## References

- Cravatt BF and Lichtman AH (2004) The endogenous cannabinoid system and its role in nociceptive behavior. *J Neurobiol* **61**:149-160.
- DeGroot JF, Coggeshall RE, and Carlton SM (1997) The reorganization of mu-opioid receptors in the rat dorsal horn following peripheral axotomy. *Neurosci Lett* **233**: 113-116.
- Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J and Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *J Neurosci* **23**:2371-2382.
- Eisenberg E, McNicol ED and Carr DB (2005) Efficacy and safety of opioid agonists in the treatment of neuropathic pain of nonmalignant origin: systematic review and meta-analysis of randomized controlled trials. *JAMA* **293**:3043-3052.
- Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Keith D Jr, Polakiewicz R and Evans CJ (2003) Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci* **23**:8360-8369.
- Farquhar-Smith WP, Egertova M, Bradbury EJ, McMahon SB, Rice AS and Elphick MR (2000) Cannabinoid CB(1) receptor expression in rat spinal cord. *Mol Cell Neurosci* **15**:510-521.

- Glaum SR, Miller RJ, and Hammond DL (1994) Inhibitory actions of delta 1-, delta 2-, and mu-opioid receptor agonists on excitatory transmission in lamina II neurons of adult rat spinal cord. *J Neurosci* **14**: 4965-4971.
- Hu HJ and Gereau RW (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. *J Neurophysiol* **90**:1680-1688.
- Ibrahim MM, Deng H, Zvonok A, Cockayne DA, Kwan J, Mata HP, Vanderah TW, Lai J, Porreca F, Makriyannis A and Malan TP Jr (2003) Activation of CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc Natl Acad Sci USA* **100**:10529-10533.
- Ibrahim MM, Porreca F, Lai J, Albrecht PJ, Rice FL, Khodorova A, Davar G, Makriyannis A, Vanderah TW, Mata HP, Malan TP (2005) CB2 cannabinoid receptor activation produces antinociception by stimulating peripheral release of endogenous opioids. *Proc Natl Acad Sci USA* **102**:3093-3098.
- Ji RR, Baba H, Brenner GJ and Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci* **2**:1114-1119.
- Ji RR, Befort K, Brenner GJ and Woolf CJ (2002) ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. *J Neurosci* **22**:478-485.
- Ji RR and Strichartz G (2004) Cell signaling and the genesis of neuropathic pain. *Sci STKE* reE14.

- Ji RR and Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol Dis* **8**:1-10.
- Jin SX, Zhuang ZY, Woolf CJ, and Ji RR (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* **23**:4017-4022.
- Karim F, Wang CC and Gereau RW (2001) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. *J Neurosci* **21**:3771-3779.
- Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ and Ji RR (2004) Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* **24**:8310-8321.
- Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**:355-363.
- Kohno T, Ji RR, Ito N, Allchorne AJ, Befort K, Karchewski LA and Woolf CJ (2005) Peripheral axonal injury results in reduced  $\mu$  opioid receptor action pre and post-synaptically in the spinal cord. *Pain* **117**:77-87.



- Lee YW, Chaplan SR and Yaksh TL (1995) Systemic and supraspinal, but not spinal, opiates suppress allodynia in a rat neuropathic pain model. *Neurosci Lett* **199**:111-114.
- Lever IJ, Pezet S, McMahon SB and Malcangio M (2003) The signaling components of sensory fiber transmission involved in the activation of ERK MAP kinase in the mouse dorsal horn. *Mol Cell Neurosci* **24**:259-270.
- Lim G, Sung B, Ji RR and Mao J (2003) Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of Win 55,212-2 on neuropathic pain behaviors in rats. *Pain* **105**:275-83.
- Mao J, Price DD, and Mayer DJ (1995) Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. *Pain* **62**:259-74.
- Molander C, Xu Q, and Grant G (1984) The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. *J Comp Neurol* **230**:133-41.
- Ossipov MH, Kovelowski CJ, Nichols ML, Hruby VJ and Porreca F (1995) Characterization of supraspinal antinociceptive actions of opioid delta agonists in the rat. *Pain* **62**:287-93.
- Ossipov MH, Lai J, King T, Vanderah TW, Malan TP, Jr., Hruby VJ and Porreca F (2004) Antinociceptive and nociceptive actions of opioids. *J Neurobiol* **61**:126-48.
- Porreca, F, Tang QB, Bian D, Riedl M, Elde R, and Lai J (1998) Spinal opioid mu receptor expression in lumbar spinal cord of rats following nerve injury, *Brain Res* **795**:197-203.

- Presley RW, Menetrey D, Levine JD and Basbaum AI (1990) Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. *J Neurosci* **10**:323-335.
- Raja SN and Haythornthwaite JA (2005) Combination therapy for neuropathic pain--which drugs, which combination, which patients? *N Engl J Med* **352**:1373-1375.
- Tsou K, Lowitz KA, Hohmann AG, Martin WJ, Hathaway CB, Bereiter DA and Walker JM (1996) Suppression of noxious stimulus-evoked expression of Fos protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist. *Neuroscience* **70**:791-798.
- Wiesenfeld-Hallin Z, Xu XJ, and Hokfelt T (2002) The role of spinal cholecystinin in chronic pain states. *Pharmacol Toxicol* **91**:398-403.
- Woolf CJ and Mannion RJ (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* **353**:1959-1964.
- Xu XJ, Puke MJ, Verge VM, Wiesenfeld-Hallin Z, Hughes and Hokfelt T (1993) Up-regulation of cholecystinin in primary sensory neurons is associated with morphine insensitivity in experimental neuropathic pain in the rat. *Neurosci Lett* **152**:129-132.
- Yoshimura M and North RA (1983) Substantia gelatinosa neurones hyperpolarized in vitro by enkephalin, *Nature* **305**:529-530.

- Zeltser R, Seltzer Z, Eisen A, Feigenbaum JJ and Mechoulam R (1991) Suppression of neuropathic pain behavior in rats by a non-psychotropic synthetic cannabinoid with NMDA receptor-blocking properties. *Pain* **47**:95-103.
- Zhang J, Hoffert C, Vu HK, Groblewski T, Ahmad S and O'Donnell D (2003) Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. *Eur J Neurosci* **17**:2750-2754.
- Zhang X, Bao L, Shi TJ, Ju G, Elde R and Hokfelt T (1998) Down-regulation of mu-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. *Neuroscience* **82**:223-240.
- Zhang Y, Conklin DR, Li X and Eisenach JC (2005) Intrathecal morphine reduces allodynia after peripheral nerve injury in rats via activation of a spinal A1 adenosine receptor. *Anesthesiology*. **102**:416-420.
- Zhao C, Tall JM, Meyer RA and Raja SN (2004) Antiallodynic effects of systemic and intrathecal morphine in the spared nerve injury model of neuropathic pain in rats. *Anesthesiology* **100**:905-911.
- Zhuang ZY, Gerner P, Woolf CJ, Ji RR (2005) ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* **114**:149-159.

## Footnotes

This study was supported by NIH grants NS40698 and TW7180 to RRJ.

## Legends for Figures

**Figure 1 (a-e).** pERK immunostaining of non-stimulated spinal cord slices (a) and stimulated slices after treatment with capsaicin (b), capsaicin plus DAMGO (c, 5  $\mu$ M), and capsaicin plus ACEA (d, 10  $\mu$ M), obtained from non-injured naive rats. Scale, 100  $\mu$ m. (e) shows colocalization between pERK and NeuN in the superficial dorsal horn after capsaicin stimulation. Scale, 20  $\mu$ m. The slices (**L4/L5**) were pretreated with DAMGO and ACEA for 10 min, followed by capsaicin stimulation (3  $\mu$ M, 5 min), and then fixed 10 min after capsaicin stimulation for pERK staining.

**Figure 2.** Quantification of pERK-IR neurons in the superficial dorsal horn (laminae I-II) of spinal slices (**L4/L5**) of non-injured naïve rats. Capsaicin (3  $\mu$ M, 5 min) induces a marked increase in the number of pERK-IR neurons, which is suppressed by DAMGO, morphine, and ACEA at certain concentrations. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , ANOVA. Mean  $\pm$  SEM (n=5).

**Figure 3 (a-d).** pERK immunostaining of non-stimulated spinal cord slices (a) and stimulated slices with treatment of capsaicin (b), capsaicin plus DAMGO (c, 5  $\mu$ M), and capsaicin plus ACEA (d, 10  $\mu$ M), obtained from nerve injured rats at one week. Scale, 100  $\mu$ m. The **L5** slices were pretreated with DAMGO and ACEA for 10 min, followed by capsaicin stimulation (3  $\mu$ M, 5 min). The dotted lines indicate the edges of the dorsal horn gray matter.

**Figure 4.** Quantification of capsaicin-induced pERK-IR neurons in the superficial dorsal horn (laminae I-II) of spinal slices from control and injured rats (one week after SNL). The **L5 or L4** spinal slices were stimulated by capsaicin (3  $\mu$ M, 5 min). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; ANOVA, n=5. Notice that capsaicin-evoked pERK expression is decreased after nerve injury. Ipsi: ipsilateral side to nerve injury. Contra: contralateral side to nerve injury.

**Figure 5.** Quantification of pERK-IR neurons in the superficial dorsal horn (laminae I-II) of **L5** spinal slices of nerve injured rats (one week after SNL). Capsaicin-induced increase in

the number of pERK-IR neurons is suppressed by ACEA but not by DAMGO and morphine. \*\*,  $P < 0.01$ , ANOVA,  $n = 5$ .

**Figure 6.** Quantification of pERK-IR neurons in the superficial dorsal horn (laminae I-II) of spinal slices (**L4**) of nerve injured rats (one week after SNL). Capsaicin-induced increase in the number of pERK-IR neurons is suppressed by ACEA, DAMGO, and morphine. \*\*,  $P < 0.01$ , ANOVA,  $n = 5$ .

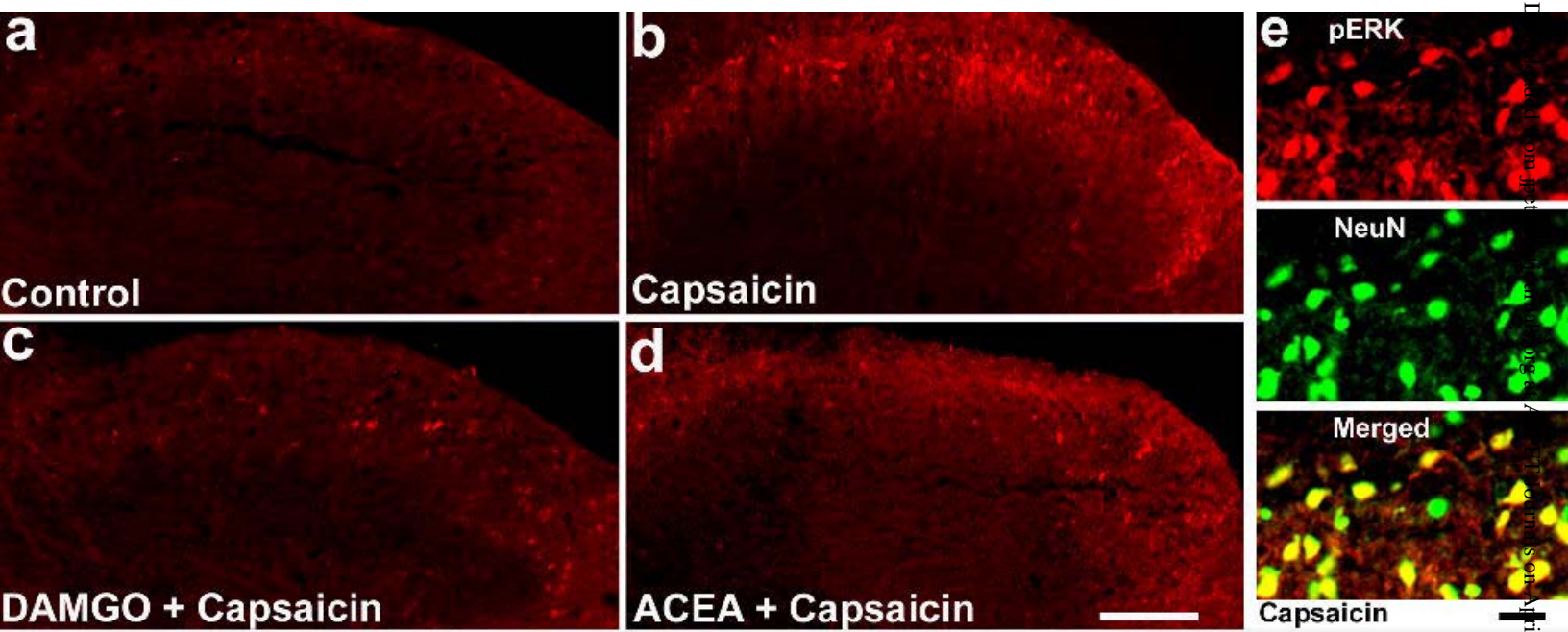
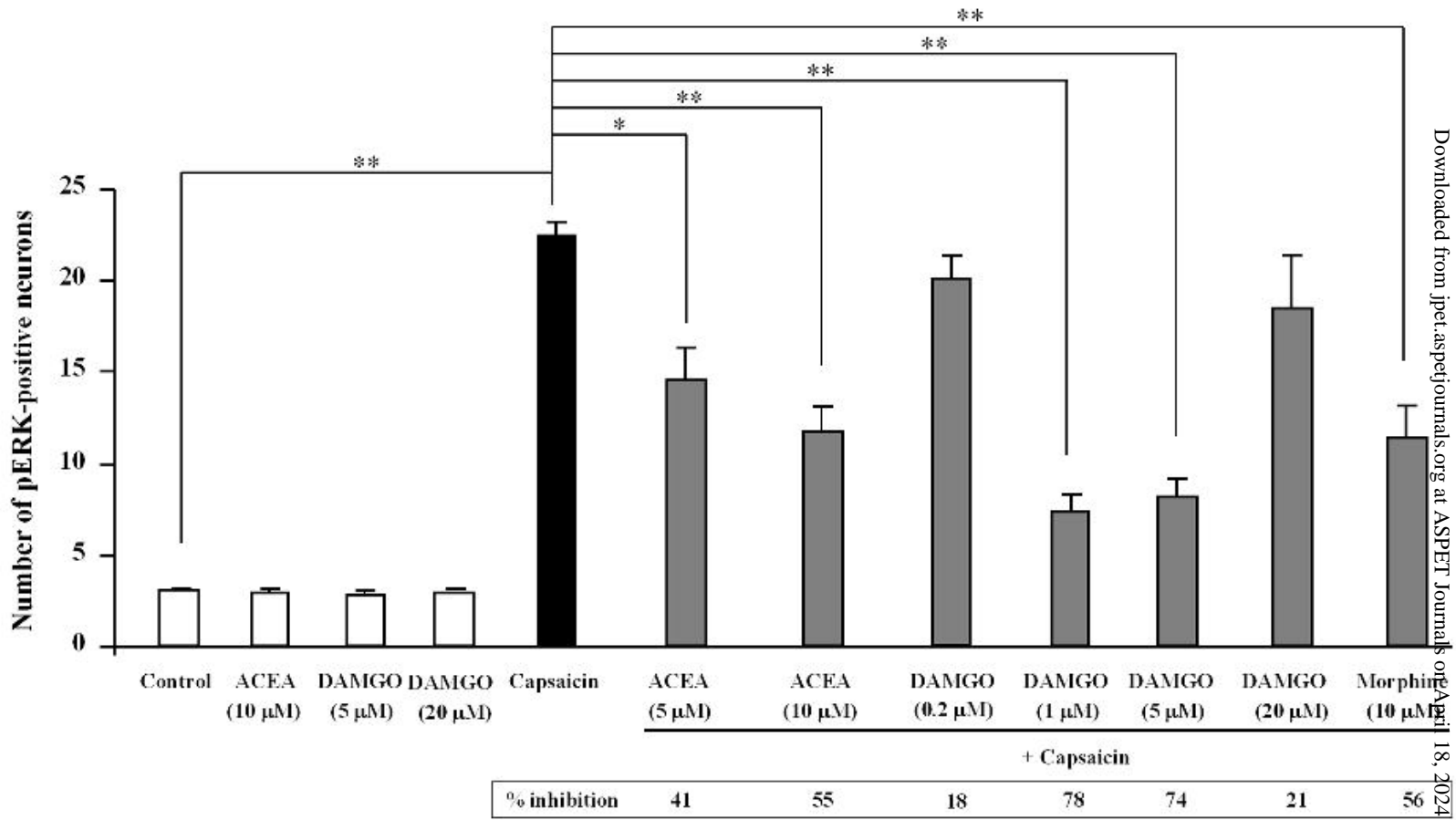
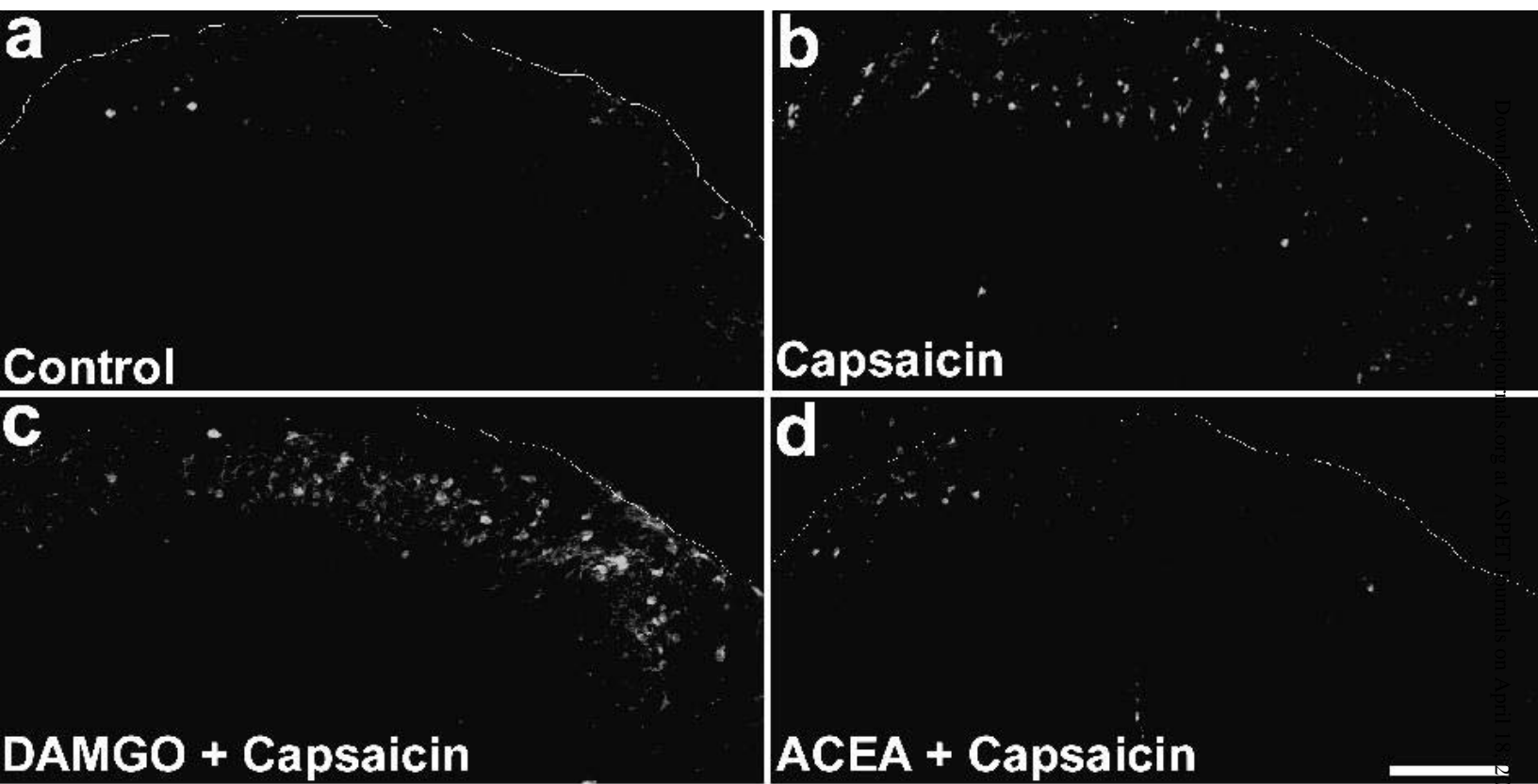


Figure 1



**Figure 2**





**Figure 3**

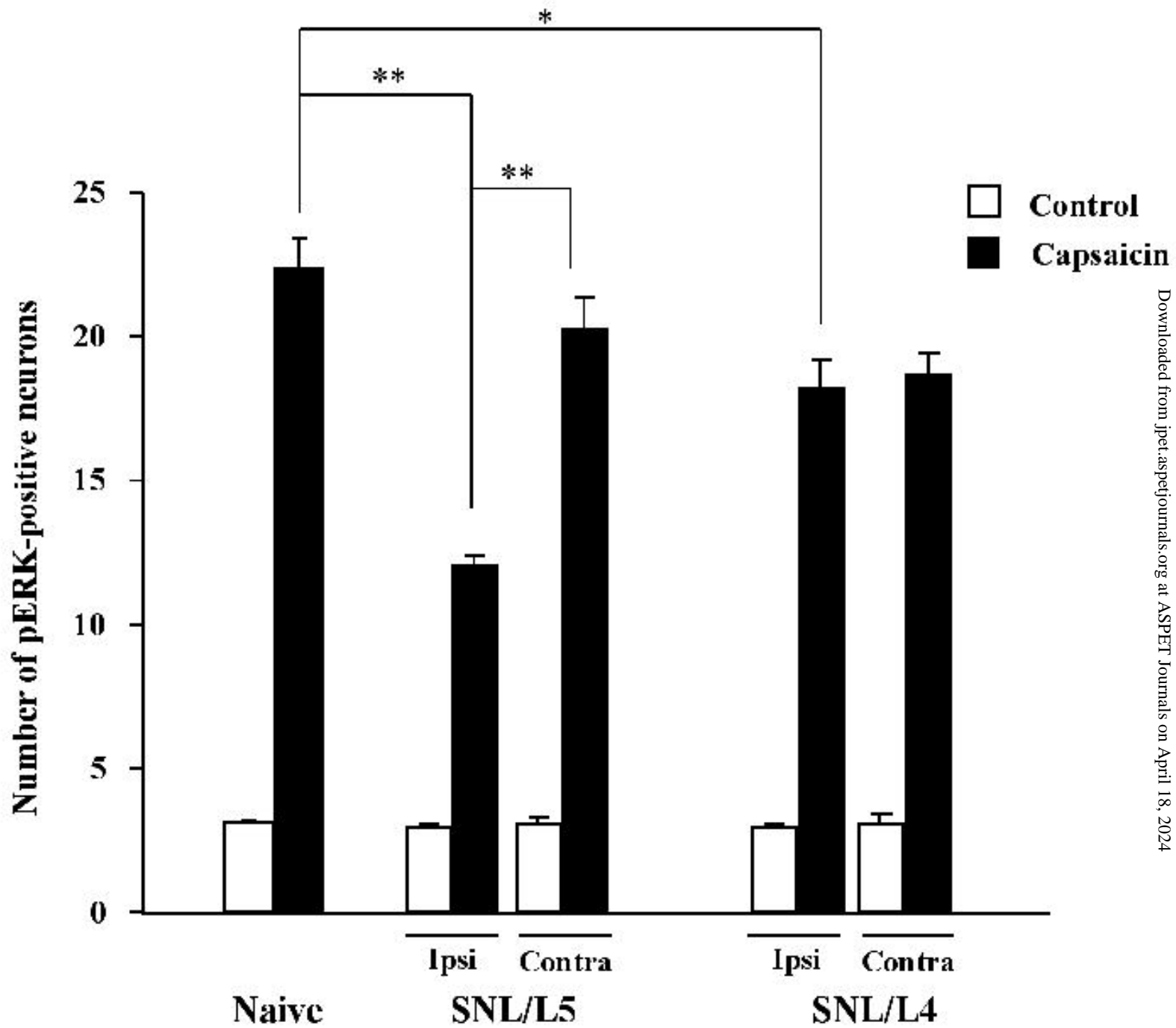


Figure 4

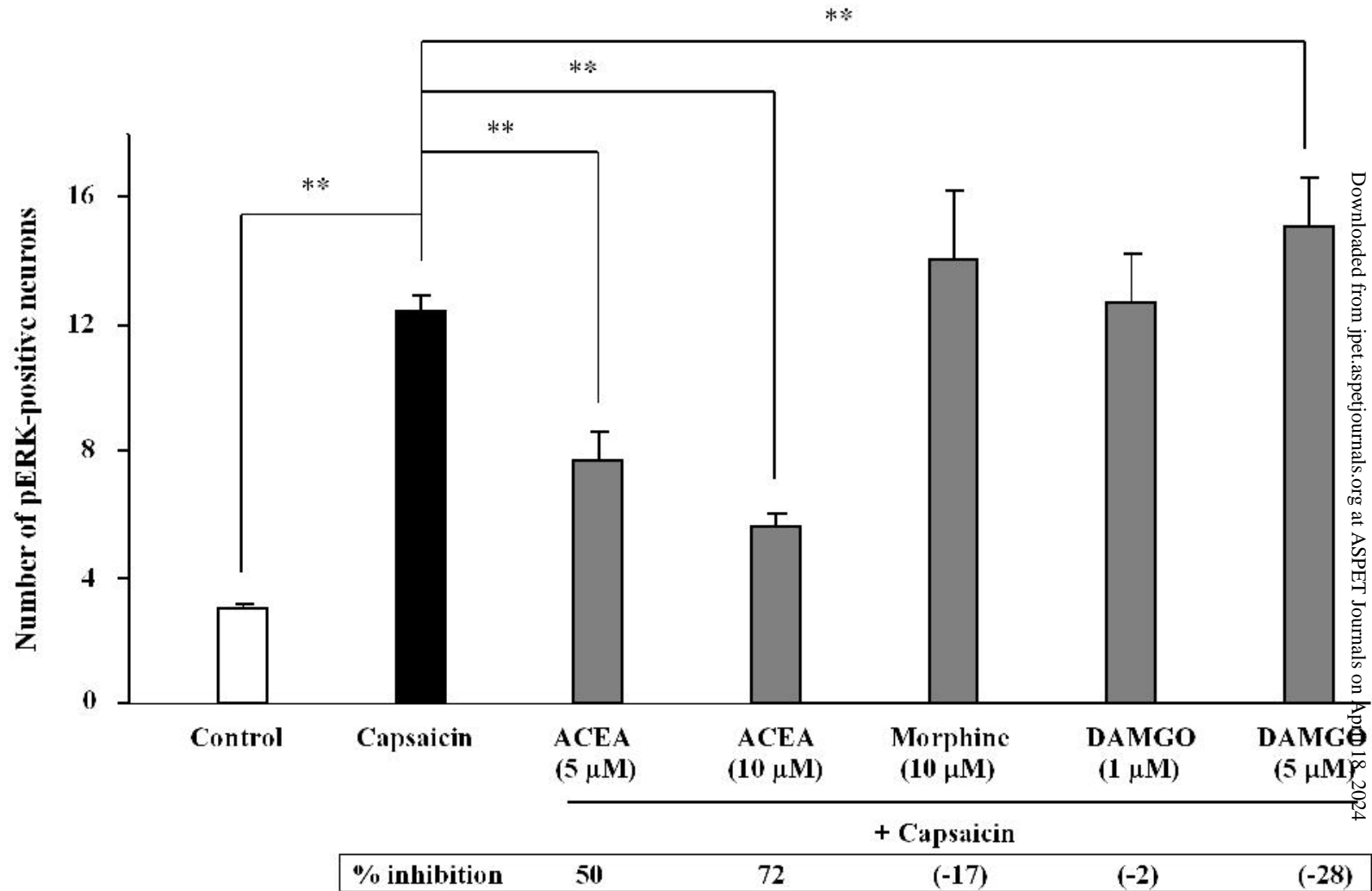
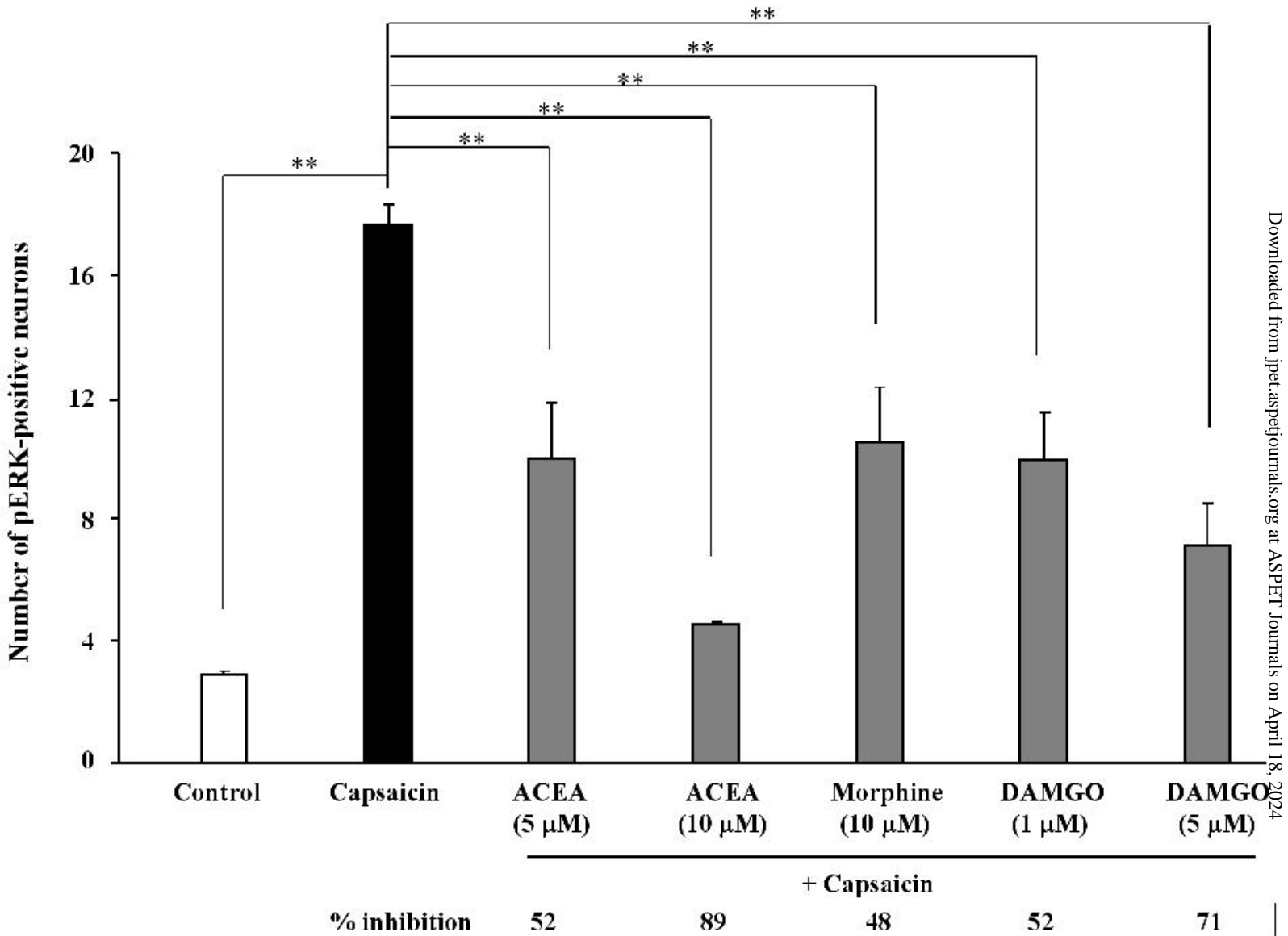


Figure 5



**Figure 6**