# Modulation of Sarcodon Aspratus on Ion Currents-induced by Excitatory Neurotransmitters in Rat Periaqueductal Gray Neurons

Sung Tae Kim, Youn Sub Kim*, Yun Hee Sung[1], Chang Ju Kim[1], Kwan Joong Joo[2], Seung Ho Han[3], Choong Yeol Lee[4]

*Department of Anatomy-Pointology, College of Oriental Medicine, Kyungwon University,*
*1: Department of Physiology, College of Medicine, Kyung Hee University,*
*2: Department of Urology, College of Medicine, Sungkyunkwan University,*
*3: Department of Physiology, College of Medicine, Eulji University, 4: Department of Physiology, College of Oriental Medicine, Kyungwon University*

Sarcodon aspratus is the mushroom of Telephoracea which has been classified into Alphllophorales. The aqueous extract of Sarcodon aspratus is known to have anti-tumor activity, immune modulatory effect, and anti-oxidative action. The descending pain control system consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla including the nucleus raphe magnus, and the spinal dorsal horn. Glutamate is the primary excitatory neurotransmitter in the brain. Glutamate receptors are divided into two main families, namely metabotropic and ionotropic receptors. Glutamate ionotropic receptors are classified as N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, and kainate receptor. In the present study, the modulation of Sarcodon aspratus on the ion currents activated by glutamate, NMDA, AMPA, and kainate in the acutely dissociated PAG neurons was investigated by nystatin-perforated patch-clamp technique under voltage-clamp condition. Sarcodon aspratus increased glutamate- and NMDA-induced ion currents, while AMPA- and kainate-induced ion currents were not increased by Sarcodon aspratus. The present results show that Sarcodon aspratus may activate the descending pain control system in rat PAG neurons through NMDA receptor.

Key words : Sarcodon aspratus, glutamate, N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, kainate

## Introduction

The descending pain control system consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla including the nucleus raphe magnus, and the spinal dorsal horn. Of these, descending pain control system is one of the most extensively studied pain control systems[1]. PAG has been identified as a key component of the pain inhibitory system since electrical or chemical stimulation of this area suppresses nociceptive transmission from the dorsal horn of the spinal cord[2].

Glutamate is the primary excitatory neurotransmitter in the brain. Abundant glutamate-binding sites are localized in the dorsolateral subdivision of the PAG[3]. Increased glutamate release in the level of PAG is known to suppress pain conduction by activation of PAG neurons during nociceptive stimulation[4]. Glutamate receptors are divided into two main families, namely metabotropic and ionotropic receptors. Glutamate ionotropic receptors are classified as N-methyl-D-aspartate (NMDA) receptor and non-NMDA receptors: kainate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. Glutamate ionotropic receptors share a common endogenous ligand and show numerous pharmacological, biochemical and modulatory differences[5].

Sarcodon aspratus is the mushroom of Telephoracea which has been classified into Alphllophorales. Sarcodon aspratus is distributed in Asia countries such as Korea, Japan, and China. Sarcodon aspratus has traditionally been used for medicinal purposes and edibility[6]. The aqueous extract of Sarcodon aspratus is known to have anti-tumor activity, immune modulatory effect, and anti-oxidative action[7].

The analgesic mechanism of Sarcodon aspratus in the context of the descending pain control system has not yet been clarified. In the present study, modulation of the aqueous extract of Sarcodon aspratus on the ion currents activated by

* To whom correspondence should be addressed at : Youn Sub Kim
Department of Anatomy-Pointology, College of Oriental Medicine, Kyungwon University, 65 San Bokjeong-dong, Sujeong-gu, Seongnam, Korea
· E-mail : ysk@kyungwon.ac.kr, · Tel : 031-750-5420
· Received : 2006/09/29 · Revised : 2006/11/01 · Accepted : 2006/11/29

glutamate, NMDA, AMPA, and kainate in the acutely dissociated PAG neurons was investigated by nystatin-perforated patch-clamp technique under voltage-clamp condition.

## Materials and Methods

1. Preparation of the PAG neurons

The PAG neurons were freshly dissociated using technique described previously elsewhere[8,9]. In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50® anesthesia (50 mg/kg; i.m.).

The brain was removed and the transverse slices (400 μm thickness) were made with a microslicer (DTK-1000, DSK, Tokyo, Japan). Slices were pre-incubated with 95% $O_2$ and 5% $CO_2$ at room temperature for 30 min. Then, the slices were treated with pronase (protease XIV, 1 mg/6 ml of the oxygenated incubation solution) for 40 - 80 min at 32℃ and subsequently with thermolysin (protease X, 1 mg/6 ml) for 10 - 20 min at 32℃. After enzyme treatment, the slices were kept in the enzyme-free incubation solution for 1 h. PAG region was identified in a 60 mm culture dish coated with silicone under a binocular microscope (SZ-ST, Olympus, Tokyo, Japan), and was micropunched out from the slicewith an electrolytically polished injection needle. The micropunched PAG regions were mechanically dissociated in a different dish with fire-polished fine glass Pasteur pipettes in 35 mm plastic culture dishes (3801, Falcon, Franklin Lake, NJ, USA) filled with standard solution. The dissociation procedure was done under an inverted phase-contrast microscope (CK-2, Olympus, Tokyo, Japan). The dissociated neurons usually adhered to the bottom of the dish within 20 min. These cells were remained viable for electrophysiological studies up to 6 h after dissociation.

2. Solutions

The ionic composition of the incubation solutions was (in mmol/l): NaCl 124, KCl 5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, glucose 10, and $NaHCO_3$ 24. The pH was adjusted to 7.4 by continuous bubbling with 95% $O_2$ and 5% $CO_2$. The composition of the standard external solution was (in mmol/l): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. The pH was adjusted to the 7.4 with tris-hydroxymethylaminomethane (Tris-Base). The composition of the internal pipette solution for nystatin-perforated recording contained (in mmol/l): KCl 150 and HEPES 10. The pH was adjusted to 7.2 by adding Tris-base. A stock solution containing 10 mg/ml nystatin was prepared and added in a final concentration of 200 ?/ml to the patch pipette solution.

3. Drugs

In order to obtain the aqueous extracts of Sarcodon aspratus, it was subsequently heat-extracted, pressure-filtered, and concentrated with a rotary evaporator. The resulting 13.22 g of powder was obtained from 50 g of Sarcodon aspratus through lyophilization by a drying machine for 24 h (yield of 26.44%).

Most drugs used in this experiment were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The following drugs were used: nystatin, glutamate, NMDA, and kainate, AMPA. Drugs were added to the standard solution at the final concentrations provided in the text and were applied using a rapid application system termed the "Y-tube method" as described elsewhere[8,9]. By this technique, the standard solution surrounding a neuron could be exchanged within 10 - 20 ms.

4. Electrical measurement

Electrical recording was performed in thenystatin-perforated patch recording mode under voltage-clamp condition. Patch pipette was prepared from glass capillaries with an outer diameter of 1.5 mm on a 2-stage puller (PB-7, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with the internal pipette solution and the reference electrode was 6 - 8 MΩ. After stable perforated patch formation, the series resistance ranged from 16 to 25 MΩ. Electrical stimulation, currents recording, and filtration of currents (at 2.9 kHz) were obtained with an EPC-7 patch-clamp amplifier (List-Electronic, Darmstadt, Germany). The currents and voltage were monitored on a pen recorder (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan). All experiments were performed at room temperature (22 - 24℃).

5. Statistical analysis

The results are presented as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. The differences were considered statistically significant at $P < 0.05$.

## Results

1. Ion currents activated by various concentrations of Sarcodon aspratus

In the nystatin-perforated patch-clamp mode, experiments were carried out at a holding potential (VH) of -50 mV. Sarcodon aspratus was applied every 2 min and ion currents activated by 0.1 mg/ml Sarcodon aspratus was used as a

control value. Inward currents induced by Sarcodon aspratus at various concentrations were recorded. The concentration of 0.001 mg/ml of Sarcodon aspratus did not elicit ion currents. The magnitude of ion currents elicited by Sarcodon aspratus at concentrations of 0.005 mg/ml, 0.01 mg/ml, and 0.05 mg/ml were 1.77 ± 0.65%, 4.09 ± 0.91%, and 40.43 ± 3.58% of the control value, respectively. In this study, it was shown that Sarcodon aspratus elicited ion currents in PAG neurons as a concentration-dependent manner (Fig. 1).

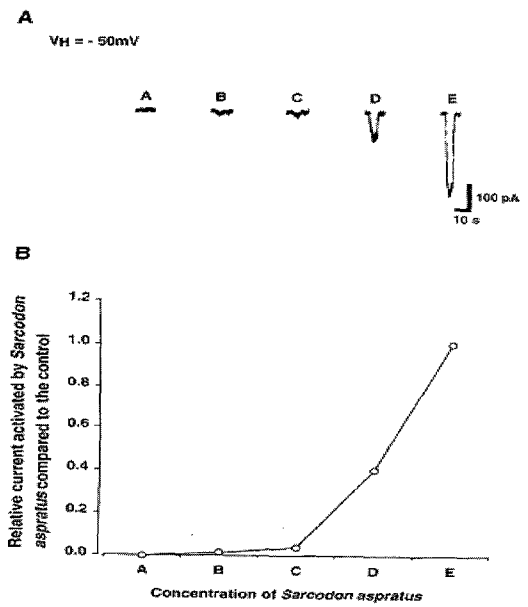


**Fig. 1. Ion currents activatedby Sarcodon aspratus.** Application of Sarcodon aspratus elicited ion currents in periaqueductal gray (PAG) neuron as a concentration-dependent manner. Sarcodon aspratus at 0.1 mg/ml was used as a control value. (A) 0.001 mg/ml Sarcodon aspratus, (B) 0.005 mg/ml Sarcodon aspratus, (C) 0.01 mg/ml Sarcodon aspratus, (D) 0.05 mg/ml Sarcodon aspratus, (E) 0.1 mg/ml Sarcodon aspratus.

2. Modulation of Sarcodon aspratus on glutamate-induced ion currents

To investigate the modulation of Sarcodon aspratus on glutamate-induced ion currents, magnitude of ion current elicited by $10^{-5}$M glutamate was used as a control value. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml was applied simultaneously with 10-5 Mglutamate, respectively. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml activated glutamate-induced ion current about 1.03 ± 0.03, 1.20 ± 0.04, and 1.67 ± 0.11 of the control value, respectively (Fig. 2). In the present results, the glutamate-induced ion current was increased significantly by 0.01 mg/ml of Sarcodon aspratus.

3. Modulation of Sarcodon aspratus on NMDA-induced ion currents

To investigate the modulation of Sarcodon aspratus on NMDA-induced ion currents, magnitude of ion current elicited by $10^{-4}$M NMDA was used as a control value. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml was applied simultaneously with $10^{-4}$ M NMDA, respectively. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml activated NMDA-induced ion current about 0.97 ± 0.04, 1.12 ± 0.09, and 1.29 ± 0.41 of the control value, respectively (Fig. 3). In the present results, the NMDA-induced ion current was increased significantly by 0.01 mg/ml of Sarcodon aspratus.

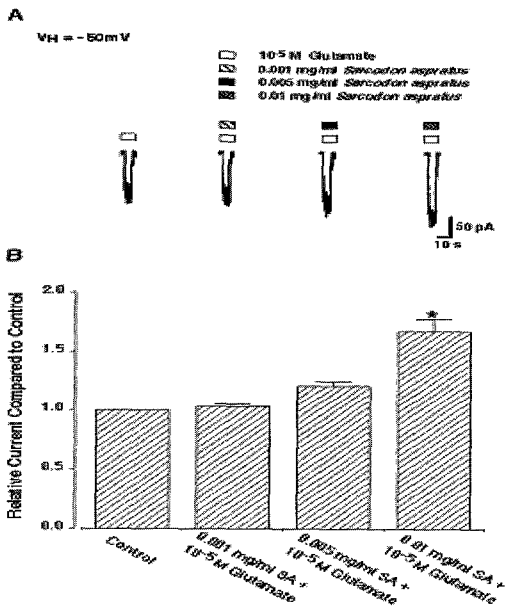


**Fig. 2. Modulation of Sarcodon aspratus on glutamate-induced ion current.** Glutamate-induced ion current was significantly activated by 0.01 mg/ml of Sarcodon aspratus. $10^{-5}$ M glutamate was used as a control value. * P <0.05 compared to the control value. SA, Sarcodon aspratus.

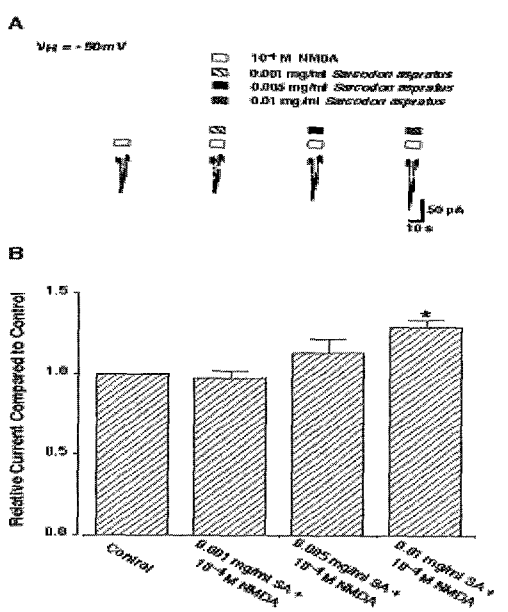


**Fig. 3. Modulation of Sarcodon aspratus on N-methyl-D-aspartate (NMDA)-induced ion current.** NMDA-induced ion current was significantly activated by 0.01mg/ml of Sarcodon aspratus. $10^{-4}$ M NMDA was used as a control value. * P <0.05 compared to the control value. SA, Sarcodon aspratus.

4. Modulation of Sarcodon aspratus on AMPA-induced ion currents

To investigate the modulation of Sarcodon aspratus on AMPA-induced ion currents, magnitude of ion current elicited by $10^{-5}$M AMPA was used as a control value. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml was applied simultaneously with $10^{-5}$M AMPA, respectively. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml activated glutamate-induced ion current about 1.07 ± 0.03, 1.12 ± 0.03, and 1.18 ± 0.06 of the control value, respectively (Fig. 4). The present results show that Sarcodon aspratus did not increased significantly AMPA-induced ion current at a concentration used in the experiment.

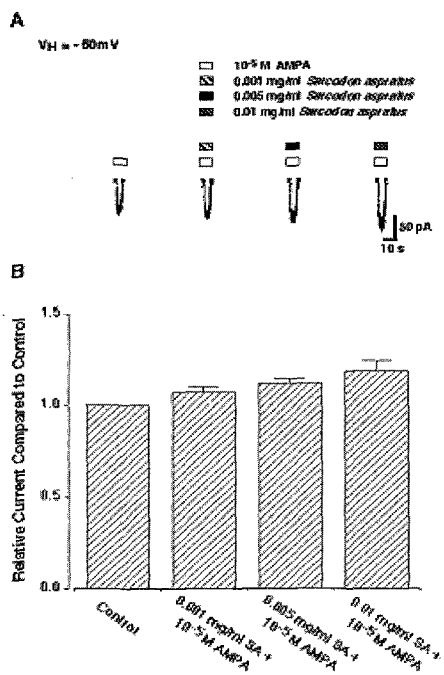


**Fig. 4. Modulation of Sarcodon aspratus on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-induced ion current.** AMPA-induced ion currents was not increased by 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml Sarcodon aspratus. $10^{-5}$ M AMPA was used as a control value. SA, Sarcodon aspratus.

5. Modulation of Sarcodon aspratus on kainate-induced ion currents

To investigate the modulation of Sarcodon aspratus on kainate-induced ion currents, magnitude of ion current elicited by $10^{-5}$M kainate was used as a control value. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml was applied simultaneously with $10^{-5}$ M kainate, respectively. Sarcodon aspratus at concentrations of 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml activated kainate-induced ion current about 1.00 ± 0.08, 1.03 ± 0.04, and 1.12 ± 0.07 of the control value, respectively (Fig. 5). The present results show that Sarcodon aspratus did not increased statistically significant in kainate-induced ion current at a concentration used in the experiment.

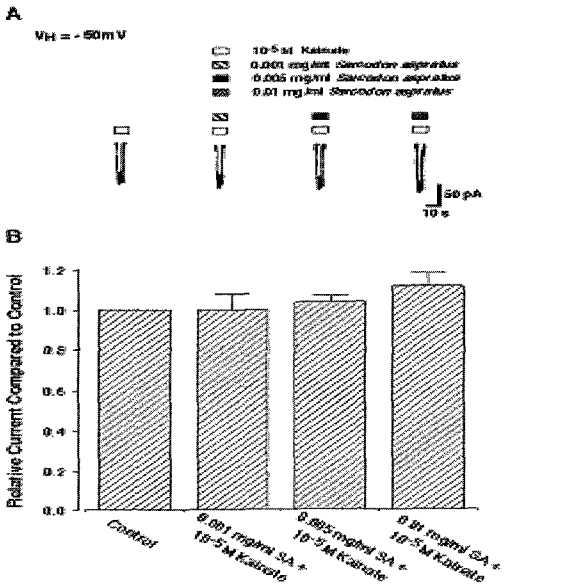


**Fig. 5. Modulation of Sarcodon aspratus on kainate-activated ion current.** Kainate-induced ion current was not increased by 0.001 mg/ml, 0.005 mg/ml, and 0.01 mg/ml Sarcodon aspratus. $10^{-5}$M kainate was used as a control value. SA, Sarcodon aspratus.

# Discussion

In the present results, Sarcodon aspratus at 0.01 mg/ml activated glutamate-induced ion current in acutely dissociated rat PAG neurons. Presynaptic release of glutamate activates various glutamate receptor that is divided into ionotropic and metabotrophic receptors. Ionotropic glutamate receptor mediates basic information processing in the brain and induces change in synaptic efficacy implicated in learning and memory, developing and maintaining cellular connections, and pain perception[10,11]. It has been reported that glutamate produces analgesic action when microinjected into the PAG area, and this effect was blocked by intra-PAG injection of glutamate antagonists, DL-AP5 and MK-801[12-14].

In the present results, Sarcodon aspratus at 0.01 mg/ml activated NMDA-induced ion current in acutely dissociated rat PAG neurons. NMDA receptor has a special characteristics such as highly $Ca^{2+}$ permeablity and a voltage-dependent $Mg^{2+}$ block. NMDA receptor is implicated in synaptic plasticity and pain transmission[15]. It has been reported that NMDA but not non-NMDA receptors located at the supraspinal level may be involved in the anti-nociception produced by morphine-induced multiplicative interactions between spine and supraspinal sites[16]. Also, it has been reported that NMDA receptor plays roles in inflammatory and neuropathic pain[17]. Therefore, it is regarded that Sarcodon aspratus concentration

of above 0.01 mg/ml may relieve pain through NMDA in acutely dissociated rat PAG neurons.

In the present results, Sarcodon aspratus did not increased significantly AMPA-induced and kainate-induced ion currents. The non-NMDA ionotropic glutamate receptor, AMPA and kainate, forms a complex with caution channels that allow passage of sodium ion, but someare also permeable to calcium depending on subunit composition[18]. Increases in postsynaptic AMPA and kainate receptors-mediated responses have been suggested to induce persistent pain conditions. Consequently, the efficacy of drugs such as AMPA receptor antagonists, kainate receptors antagonist, as well as mixed AMPA and kainate receptor antagonists in controlling pain might be enhanced during persistent pain[19]. As results, it is suspeced that low concentration (below 0.01mg/ml) Sarcodon aspratus may alleviate pain through AMPA, kainate in acutely dissociated rat PAG neurons.

Mushrooms are widely consumed not only as foods but also as alternative medicine. Mushrooms like Sarcodon aspratus contain polysaccharides, terpenoids, steroids, provitamin D, and ergosterol. Of these, steroids are abundantly present in mushrooms[20,21] and were reported to have diverse physiological functions, such as anti-oxidant effect, anti-inflammatory activity, andinhibitory effects on cancer cell growth[20-22]. Inflammation is a complex process which commences with a primary reaction in tissues. Pain is one of the signs of inflammation, and it is important clues of immunological activity[23]. Accordingly, it is suspected that steroids, components of Sarcodon aspratus, chiefly have an effect on pain inhibition.

Here in this study, we have shown that modulation of Sarcodon aspratus on NMDA receptor may activate the descending pain inhibitory system in rat PAG neurons

## Acknowledgements

This work was supported by a grant (Code #20050301034457) from BioGreen 21 Program, Rural Development Administration, Republicof Korea.

## References

1. Fields, H.L., Heinricher, M.M., Mason, P. Neurotransmitters in nociceptive modulatory circuits. Annu Rev Neurosci 14: 219-245, 1991.
2. Basbaum, A.I., Fields, H.L. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 7: 309-338, 1984.
3. Greenamyre, J.T., Young, A.B., Penney, J.B. Quantitative autoradiographic distribution of L-[$^3$H] glutamate-binding sites in rat central nervous system. J Neurosci 4: 2133-2144, 1984.
4. Silva, E., Hernandez, L., Contreras, Q., Guerrero, F., Alba, G. Noxious stimulation increases glutamate and arginine in the periaqueductal gray matter in rats: a microdialysis study. Pain 87: 131-135, 2000.
5. Dingledine, R., Borges, K., Bowie, D., Traynelis, S.F. The glutamate receptor ion channels. Pharmacol Rev 51: 7-61, 1999.
6. Kim, J.W., Moon, B.S., Park, Y.M., Yoo, N.H., Yoo, I.J., Yoo, I.D., Kim, J.P., Nguyen, T.C. Structures and Antioxidant Activity of Diketopiperazines Isolated from the Mushroom Sarcodon aspratus. J Korean Soc Appl Biol Chem 48: 93-97, 2005.
7. Song, J.H., Lee, H.S., Hwang, J.K., Han, J.W., Ro, J.G., Keum, D.H., Park, K.M. Physiological activity of Sarcodon aspratus Extracts. Korean J Food Sci Ani Resour 23: 172-179, 2003.
8. Han, S.H., Cho, Y.W., Kim, C.J., Min, B.I., Rhee, J.S., Akaike, N. Mu-opioid agonist-induced activation of G-protein-coupled inwardly rectifying potassium currents in rat periaqueductal gray neurons. Neuroscience 90: 209-219, 1999.
9. Kim, Y., Shin, M., Chung, J., Kim, E., Koo, G., Lee ,C., Kim, C. Modulation of Chelidonii herba on GABA activated chloride currents in rat PAG neurons. Am J Chin Med 29: 265-279, 2001.
10. Bliss, T.V.P., Collingridge, G.L. A synaptic model ofmemory: long-term potentiation in the hippocampus. Nature 361: 31-39, 1993.
11. Woolf, C.J., Salter, M.W. Neuronal plasticity: increasing the gain in pain. Science 288: 1765-1769, 2000.
12. Urca, G., Nahin, R.L., Liebeskind, J.C. Glutamate-induced analgesia: blockade and potentiation by naloxone. Brain Res 192: 523-530, 1980.
13. Maione, S., Marabese, I., Leyva, J., Palazzo, E., De Novellis, B., Rossi, F. Characterization of mGluRs which modulate nociception in the PAG of the mouse. Neuropharmacology 37: 1475-1483, 1998.
14. Jensen, T.S., Yaksh, T.L. Brainstem excitatory amino acid receptors in nociception microinjection mapping and pharmacological characterization of glutamate sensitive sites in the brainstem associated with algogenic behavior. Neuroscience 46: 535-547, 1992.
15. Coderre, T.J., Katz, J., Vaccarino, A.L., Melzack, R. Contribution of central plasticity to pathological pain: review of clinical and experimental evidence. Pain 52: 259-285, 1993.
16. Suh, H.W., Song, D.K., Choi, Y.S., Kim, Y.H. Multiplicative interaction between intrathecally and intracerebroventricularly administered morphine for antinociception in the mouse:

Involvement of supraspinal NMDA but not non-NMDA receptors. Life Sci 56: 181-185, 1995.

17. Hizue, M., Pang, CH., Yokoyama, M. Involvement of N-methyl-D-aspartate-type glutamate receptor epsilon1 and epsilon4 subunits in tonic inflammatory pain and neuropathic pain. Neuroreport 16: 1667-1670, 2005.
18. Hollmann, M., Hartley, M., Heinemann, S. $Ca^{2+}$permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 252: 851-853, 1991.
19. Zhuo, M. Silent glutamatergic synapses and long-term facilitation in spinal dorsal horn neurons. Prog Brain Res 129: 101-113, 2000.
20. Urban, M.O., Coutinho, S.V., Gebhart, G.F. Involvement of excitatory amino acid receptors and nitric oxide in the rostral ventromedial medulla in modulating secondary hyperalgesia produced by mustard oil. Pain 81: 45-55, 1999.
21. Yasukawa, K., Aoki, T., Takido, M., Ikekawa, T., Saito, H., Matsuzawa, T. Inhibitory effects of ergosterol isolated from the edible mushroom Hypsizigus marmoreus on TPA-induced inflammatory ear oedema and tumour promotion in mice. Phytother Res 8: 10-13, 1994.
22. Bok, J.W., Lermer, L., Chilton, J., Klingeman, H.G., Towers, G.H. Antitumor sterols from the mycelia of Cordyceps sinensis. Phytochemistry 51: 891-898, 1999.
23. Zhang, Y., Mills, G.L., Nair, M.G. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom Grifola frondosa. J Agric Food Chem 50: 7581-7585, 2002.