대 한 체 질 인 류 학 회 지
Korean J Phys Anthropol
18(4): 303 ~ 312, 2005

# Changes of Neuropathic Pain in nNOS Knock-out Mice

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Changes in nitric oxide production in spinal cord or dorsal root ganglion have been known to contribute to allodynia after nerve injury. However, regulation of nNOS expression was also reported not to be responsible for the development and/or maintenance of neuropathic allodynia. The aim of this study was to elucidate role of nNOS expression in the sensory nervous system in neuropathic pain.

Von Frey and acetone tests were performed in a model of peripheral neuropathy, ligation of 5th lumbar and 6th lumbar spinal nerves, in wild type and nNOS (-/-) mice. The effect of nNOS inhibitor was evaluated in neuropathic pain behavior in the mice.

Mechanical allodynia was slightly reduced in nNOS (-/-) mice compared with wild type mice after peripheral neuropathy. nNOS inhibitor, L-NAME, reduced minimally mechanical allodynia, not cold allodynia, but gabapentin reduced remarkably neuropathic pain behavior (mechanical and cold allodynia) in both wild type and nNOS (-/-) mice.

These results suggested that nNOS expression in the sensory nervous system may be partially associated with development and/or maintenance of mechanical allodynia in a mouse model of peripheral neuropathy.

**Key words :** nNOS, Neuropathic pain, Allodynia, Nitric oxide

## Introduction

Peripheral nerve injury may lead to a chronic neuropathic pain state that is characterized by spontaneous ongoing pain, pain resulting from stimuli that are normally innocuous (allodynia), and increased pain to suprathreshold stimuli (hyperalgesia). Several models of neuropathic pain in the rat have been developed in an attempt to determine the mechanisms of neuropathic pain (Bennet and Xie 1988, Seltzer et al. 1991, Kim and Chung 1992). The sensory abnormalities in these models mimic those often observed in patients with chronic pain following peripheral nerve injuries. The literature on the subject from various animal models offers numerous possible pathophysiological scenarios: peripheral sensitization of A-delta/C-fibers, awakening of silent nociceptors, phenotype switch of A-beta fibers, loss of A-beta mediated inhibition in the dorsal horn of the spinal cord, central sensitization, sprouting of mechenoreceptive fibers in the dorsal horn, and tonic activation of descending facilitation of spinal circuitary from the brainstem (Hansson 2003).

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Nitric oxide (NO) is a short-lived, unstable free radical generated in many mammalian cells. It has a role as an intracellular messenger in different biological processes such as neurotransmission and glutamate-mediated neurotoxicity, in regulation of vascular resistance and blood pressure (Sessa et al. 1992, Nunokawa et al. 1993, Dawson and Dawson 1996). NO can be indirectly determined by demonstrating the presence of nitric oxide synthase (NOS), the enzyme that generates NO from L-arginine. The activity of all known subtype (nNOS, eNOS, iNOS) is dependent on NADPH, flavin mononucleotide (FMN), and flavin-adenin dinucleotide (FAD), therefore, the activity of NOS has often measured cytochemically as NADPH-diaphorase (NADPH-d) activity (Bredt et al. 1991, Dawson et al. 1991).

Changes in NO production in spinal cord or dorsal root ganglia (DRG) may contribute to allodynia after nerve injury (Cizkova et al. 2002). NO release in spinal cord or DRG is evoked by N-methyl-D-aspartate (NMDA) receptors activation (Selzer et al. 1991, Kristensen et al. 1992, Mao et al. 1993). NO has been shown to enhance the release of excitatory amino acids (Meller et al. 1992, McMahon et al. 1993). Pharmacological evidence regarding the role of spinal NO in the development of nerve injury-evoked allodynia has been conflicting. Some investigators observed allodynia inhibition in nerve-injured rats after treatment L-$N^G$-nitro-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor (Meller et al. 1993, Levy and Zochodne 1998, Yoon et al. 1998). Other investigators (Calcutt and Chaplan 1997, Luo et al. 1999) reported that nNOS regulation in DRG neurons may play an important role in neuroplasticity, not in neuropathic pain, after nerve injury.

Previous studies have indicated that NOS-labeled cells increase in the ipsilateral DRG following sciatic nerve transactions of L5 and L6 spinal nerves (Fiallos-Estrada et al. 1993, Steel et al. 1994). In addition, peripheral nerve section results in increase in NOS mRNA in the corresponding DRG (Verge et al. 1992). A model of nerve-injury-induced pain demonstrates that ipsilateral decrease in paw withdrawal threshold is associated with an increase in total NOS activity in the DRG, which show that NOS activity are relevant to the genesis and/or maintenance of altered pain behavior (Choi et al. 1996). However, Luo et al. (1999) reported that systemic treatment with a specific pharmacological inhibitor of nNOS failed to prevent or reverse allodynia in nerve-injured rats. They insisted that upregulation of nNOS is not responsible for the development and/or maintenance of allodynia after nerve injury.

The aim of this study was to elucidate role of nNOS expression in the sensory nervous system in neuropathic pain. We compared neuropathic pain behavior in a model of peripheral neuropathy between wild type and nNOS knock-out [nNOS (-/-)] mice.

## Materials and Methods

### 1. Experimental animals

Twelve male wild type C57BL/6 (3-4 months old) and 12 male nNOS (-/-) (3-4 months old), B6;129S4-$Nos1^{tm1Plh}$, mice were used in this experiment. The nNOS (-/-) mice were obtained from Dr. Lee (Induced Mutant Resources Program, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). They were maintained under the standard laboratory conditions on a 12-hour light/dark cycle, with free access to food and water. All animal-related procedures and care were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology.

### 2. Surgery for mouse neuropathic pain model

A unilateral peripheral neuropathy was induced

according to the modified method previously described (Kim and Chung 1992). Briefly, under gaseous anesthesia with a mixture of halothane and 1 : 2 flow ratio of $N_2O/O_2$, the left $5^{th}$ lumbar and $6^{th}$ lumbar spinal nerves were isolated and cut. Hemostasis was confirmed and the wound was then sutured.

### 3. Behavioral test in wild type and nNOS (−/−) mice

From one to four weeks after surgery, frequency of foot withdrawal in response to normally innocuous mechanical stimuli was measured. Mechanical stimuli were applied with a set of von Frey filaments ranging from 8.4 to 186.7 mN (8.4, 13.5, 24.5, 54.4, 100.5, and 186.7 mN) to evaluate mechanical allodynia. The mice were placed on a metal mesh floor covered by a transparent plastic dome, and von Frey filaments were applied from underneath the metal mesh floor to the plantar surface of the foot. The von Frey filament was applied 10 times (once every 3−4 s) to each paw. The occurrence of foot withdrawal was expressed as a percentage (number of trials accompanied by foot withdrawal/10 × 100 = % frequency of foot withdrawal). After von Frey test, cold acetone solution were applied to evaluate cold allodynia. Acetone was applied from underneath the metal mesh floor to the plantar surface of the foot. Acetone was applied 5 times (once every 5 min) to each paw. The occurrence of foot withdrawal was expressed as a percentage (number of trials accompanied by foot withdrawal/5 × 100 = % frequency of foot withdrawal).

### 4. Behavioral changes in drug administration

After behavioral testing (von Frey and acetone tests), the NOS inhibitor L−nitro−arginine−methyl ester (L−NAME) (100, 200, and 300 μmol/kg, n = 6, Sigma, USA) and gabapentin (100 mg/kg, n = 6, Sigma, USA), a variety of new antiepileptic drugs which is now used for treatment of neuropathic pain and migrane (Pappagallo 2003), were administered intraperitoneally to the wild type and nNOS (−/−) mice. Foot withdrawal frequencies were measured by the von Frey filament and acetone tests 30 min after drug administration.

### 5. Tissue preparation for immonohistochemistry for nNOS

Seven days after neuropathic surgery, the animals were subjected to pentothal sodium anesthesia (50 mg/kg, i.p.) and transcardiac perfusion fixation with saline followed by 4% phosphated−buffered paraformaldehyde. The spinal cords were obtained, post−fixed in cold 4% paraformaldehyde solution, and then placed in a 30% sucrose solution at 4°C overnight. Frozen sections (30 μm thick) of each tissue were prepared and collected in Phosphated buffered solution (PBS) in 24−well plates.

### 6. Immunohistochemistry for nNOS

To perform immunohistochemistry for nNOS, the tissue sections were immersed for 30 min in 3% $H_2O_2$ to inactivate endogenous peroxidases. Sections were incubated 1 hour at room temperature (RT) in the polyclonal nNOS antibody (1 : 250, Santa Cruz, USA) in 0.1 M PPS, pH 7.4, containing 0.1% Triton X−100, 1.5% bovine serum albumin (BSA), and 1 : 200 normal goat serum (NGS), followed by incubation for 1 hour at RT in 1 : 200 biotinylated goat anti−rabbit IgG (Vector, USA) and 1 : 200 NGS in PBS. Immnunoreactions were visualized by incubation for 1 hour at RT in avidin−biotin−peroxidase complex (1 : 100, ABC kit, Vector, USA) in PBS and 5−10 min in 0.05% 3,3′−diaminobenzidine (DAB) and 0.01% $H_2O_2$ in 0.1 M PBS.

### 7. Data analysis

The foot withdrawal frequencies were expressed as mean ±S.E.M. The differences were analyzed by the paired t−test. $P < 0.05$ was considered to be statistically

significant.

## Results

### 1. Confirmation of wild type and nNOS (−/−) mice

To confirm wild type and nNOS (−/−) mice used in this experiment, immunohistochemistry for nNOS was performed in the spinal cords of wild type and nNOS (−/−) mice. nNOS immunoreactive neurons were found around central canal (Fig. 1A) and in the lamina II and III of the dorsal horn in the spinal cord (Fig. 1C) of wild type mice. However, nNOS immunoreactive neuron was not found around central canal (Fig. 1B) and in the dorsal horn (Fig. 1D) of the nNOS (−/−) mice. Therefore, nNOS (−/−) mice used in this experiment were conformed as exact nNOS (−/−) mice.

### 2. Comparison of neuropathic pain behavior between wild type and nNOS (−/−) mice

Foot withdrawal frequency to von Frey test was about 77% one week after the nerve injury, and then slightly decreased 2 and 4 weeks after the nerve injury in wild type mice. Foot withdrawal frequency to von Frey test was 60% to 57% from one to four weeks after the nerve injury in nNOS (−/−) mice (Fig. 2). Foot withdrawal frequency to von Frey test in wild type mice was higher than that in nNOS (−/−) mice one and two weeks after the nerve injury ($p < 0.05$).

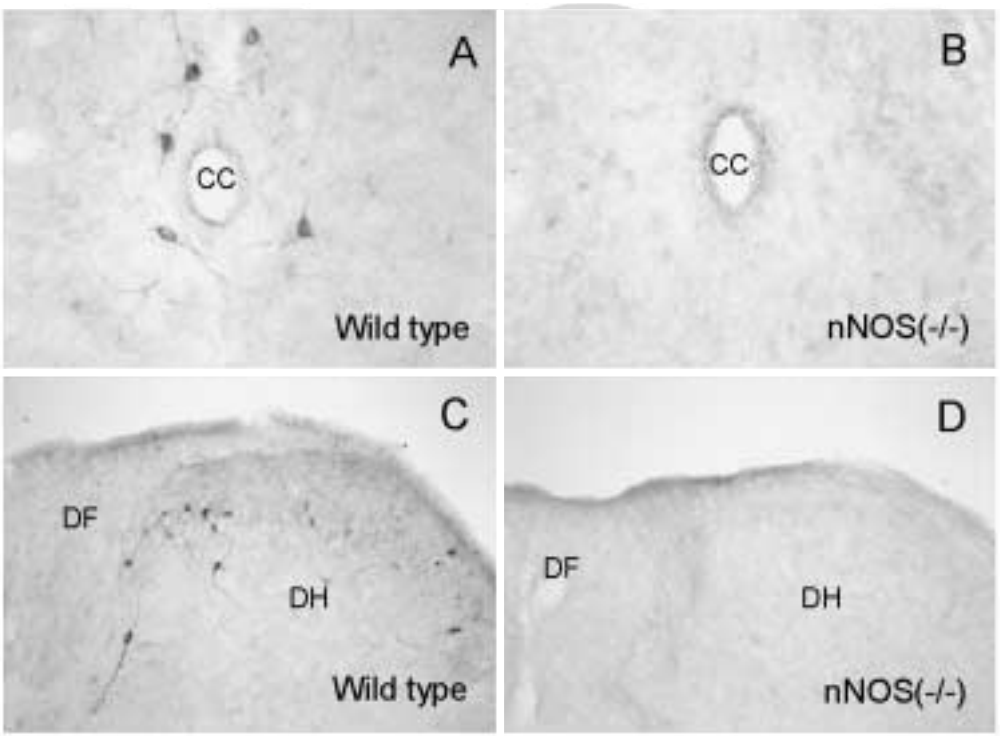


**Fig. 1.** nNOS immunoreactivities in the spinal cords in wild type and nNOS (−/−) mice (original magnification: A, B: ×400, C, D: ×200), nNOS immunoreactive cells are found around the central canal (CC) (A) and in the dorsal horn (DH) (C) of wild type mice. However, there was no nNOS immunoreactive cells in the spinal cord of nNOS (−/−) mice (B and D). DF: dorsal funiculus

Foot withdrawal frequency to acetone test was around 90% in wild type mice, 80% in nNOS (−/−) mice, from one to four weeks after nerve injury. However, Foot withdrawal frequency to acetone test in wild type mice was not statistically different from that in nNOS (−/−) mice after the nerve injury (Fig. 3).

These data show that mechanical allodynia was reduced in nNOS (−/−) mice compared with wild type mice in early phase of neuropathic pain. However, cold allodynia was not different between wild type and nNOS (−/−) mice.

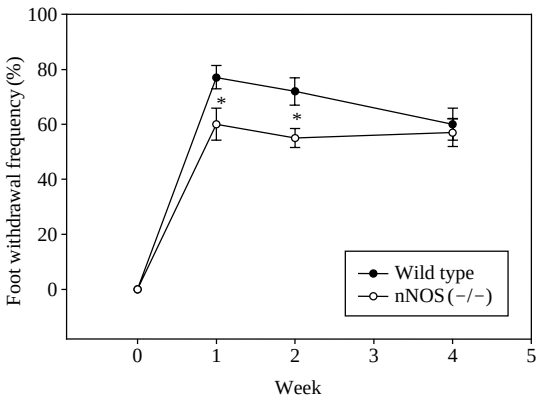


**Fig. 2.** Foot withdrawal responses to von Frey filament in wild type (n = 6) and nNOS (−/−) (n = 6) mice, * Statisitcally significantly different

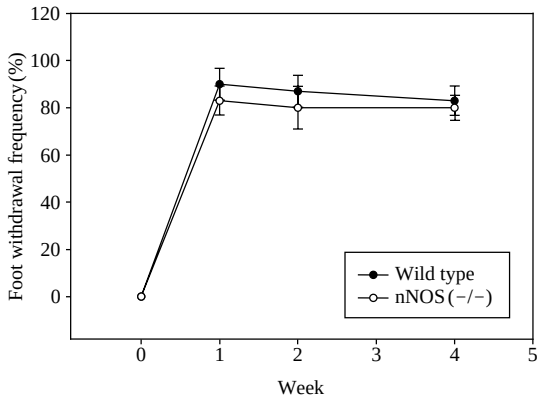


**Fig. 3.** Foot withdrawal responses to acetone test in wild type (n = 6) and nNOS (−/−) (n = 6) mice

## 3. Pharmacological responses of allodynia with L−NAME or gabapentin

Intraperitoneal injection of L−NAME (100 μmol/kg) did not reduced foot withdrawal frequency to von Frey and acetone tests 30 min later in both wild type

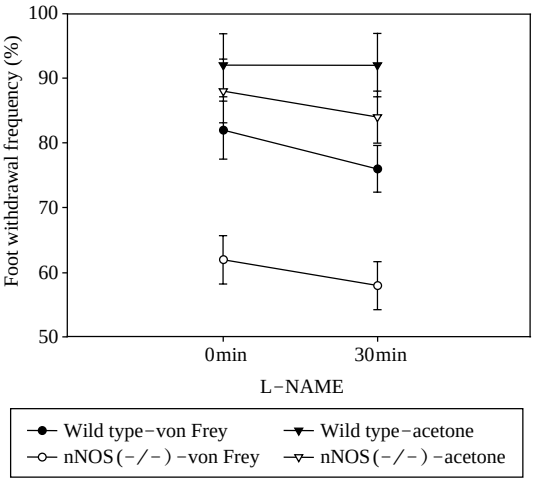


**Fig. 4.** Foot withdrwal responses to L−NAME (100 mol/kg) in wild type (n = 6) and nNOS (−/−) (n = 6) mice

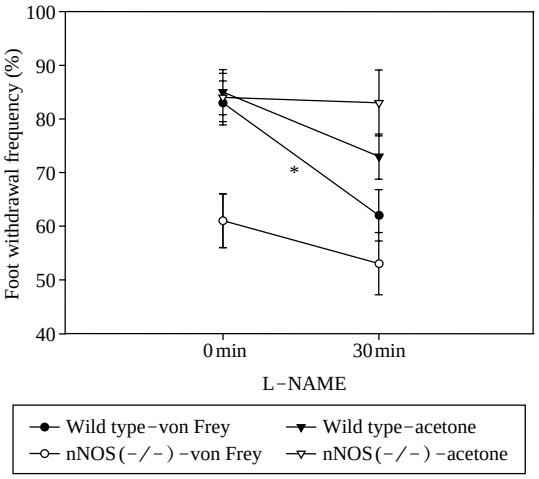


**Fig. 5.** Foot withdrwal responses to L−NAME (300 μmol/kg) in wild type (n = 6) and nNOS (−/−) (n = 6) mice, *statistically significantly different

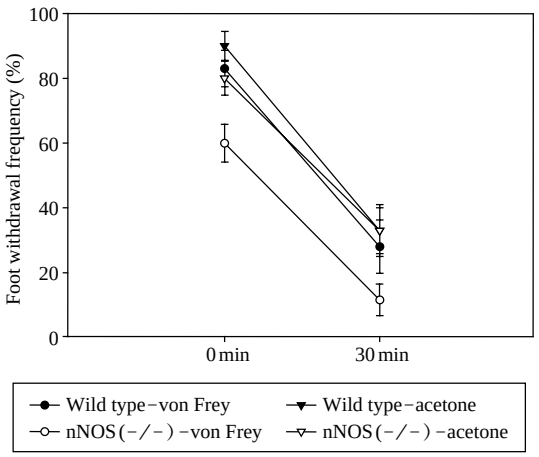


**Fig. 6.** Foot withdrawal responses to gabapentin (100 mg/kg) in wild type (n = 6) and nNOS (−/−) (n = 6) mice, Foot withdrawal frequencies in each group were statistically significantly decreased 30 min after gabapentin administration.

and nNOS (−/−) mice (Fig. 4). Intraperitoneal injection of L-NAME (200 μmol/kg) also failed to reduce foot withdrawal frequency in both wild type and nNOS (−/−) mice (data not shown). However, intraperitoneal injection of L-NAME (300 μmol/kg) slightly reduced foot withdrawal frequency to von Frey and acetone tests 30 min later in only wild type mice ($p < 0.05$, Fig. 5). Intraperitoneal injection of gabapentin (100 mg/kg) reduced markedly foot withdrawal frequency to von Frey and acetone tests 30 min later in both wild type and nNOS (−/−) mice ($p < 0.01$, Fig. 6).

These data show that nNOS inhibitor, L-NAME, reduced minimally neuropathic pain behavior in wild type mice, but gabapentin reduced remarkably neuropathic pain behavior in both wild type and nNOS (−/−) mice.

## Discussion

The present study showed that mechanical allodynia was reduced in nNOS (−/−) mice compared with wild type mice. A nNOS inhibitor, L-NAME, minimally reduced neuropathic pain behavior, but gabapentin minimally reduced neuropathic pain behavior in wild type and nNOS (−/−) mice.

A model of nerve-injury-induced pain demonstrated that an ipsilateral decrease in paw withdrawal threshold was associated with an increase in total NOS activity in the DRG. The changes in NOS activity in the DRG were observed 2 weeks after the nerve injury at the level of the injured spinal nerves but not in neighboring DRGs corresponding to uninjured spinal nerves. These data may give a clue that local changes in NOS activity are relevant to the genesis and/or maintenance of altered pain behavior (Choi et al. 1996).

Mechanical allodynia was suppressed by L-NAME (200, 100, 50, 10 μmol/kg, i.p.), in a dose-dependent manner in an experimental neuropathic pain model (6-7 weeks old Sprague-Dawley rats) (Yoon et al. 1998). Previous reports (Meller et al. 1992, Salter et al. 1996, Yamamoto and Shimoyama 1996) supported the hypothesis that NO acts in the spinal cord. In this model of neuropathic pain, neuropathic pain behaviors were reduced both by a NMDA receptor antagonist and a NOS inhibitor (Qian et al. 1996, Chaplan et al. 1997). This raised a possibility that NMDA receptor-mediated NO production might be involved in generating persistent abnormal pain. On the other hand, several lines of evidence raise the possibility that L-NAME acts on peripheral sites: the ectopic discharges recorded in the DRG in this model (Steel et al. 1994, Choi et al. 1996), and L-NAME infusion directly on the injured nerve blocked the development of heat hyperalgesia in the chronic constrictive injury to the sciatic nerve in rats (Thomas et al. 1996).

Luo et al. (1999) reported that systemic treatment with a specific pharmacological inhibitor of nNOS, 7-nitroindazole (7-NI) (50 mg/kg), failed to prevent or reverse allodynia in nerve-injured Sprague-Dawley rats. Upregulation of nNOS is not responsible for the development and/or maintenance of allodynia after nerve injury. Even though upregulation of nNOS

mRNA precedes the onset of allodynia and persists for the duration of the neuropathic pain state in nerve−ligated animals, their study indicate a clear separation between nNOS upregulation and the development and maintenance of tactile allodynia after nerve injury.

Nerve−ligated Holzman rats, which do not develop allodynia, show a remarkable nNOS upregulation in the DRG (Luo et al. 1999). Severe neuropathic pain behavior may be result from increase of NOS activity in the DRG of young (5 weeks old) rats after spinal nerve ligation, whereas NO production may be not significantly related to neuropathic pain behavior in spinal nerve ligation model of adult (16 weeks old) rats (Han et al. 2002). These data show that mechanism of neuropathic pain could be different according to the strain or age of experimental animals. The present study showed that frequency of mechanical and cold allodynia and drug response were different in wild type and nNOS (−/−) mice. These results suggest that mechanism of neuropathic pain may be also different according to modalities of pain such as mechanical and cold allodynia (Andrew and Greenspan 1999, Attal et al. 2004).

The present study showed that mechanical allodynia was slightly reduced nNOS (−/−) mice compared with wild type mice after peripheral neuropathy, and a high dose of NOS inhibitor reversed slightly mechanical allodynia in the wild type mice. These data suggest that nNOS expression in the sensory nervous system, including DRG, spinal cord, and other central nervous system related to pain pathway, is partially responsible for the development and/or maintenance of mechanical allodynia after spinal nerve injury.

Gabapentin is an anticonvulsant, structurally related to the neurotransmitter γ−aminombutyric acid (GABA). Gabapentin has antihyperalgesic and antiallodynic properties, but does not have significant actions as an anti−nociceptive agent. Its mechanisms of action appear to be a complex synergy between increased GABA synthesis, non−NMDA receptor antagonism and binding to the $\alpha_{2\delta}$ subunit of voltage dependent calcium channels. The latter action inhibits the release of excitatory neurotransmitters. Clinically, several large randomized controlled trials have demonstrated its effectiveness in the treatment of a variety of neuropathic pain syndromes (Fox et al. 2003, Bennett and Simpson 2004, Levendoglu et al. 2004).

Intraperitoneal injection of gabapentin significantly alleviated mechanical, warm and cold allodynia in a dose−dependent manner in a rat model of peripheral neuropathy (Back et al. 2004). Gabapentin reverses mechanical allodynia induced by sciatic nerve ischemia and formalin−induced nociception in mice (Gustafsson et al. 2003). The present study showed that gabapentin reduced remarkably mechanical and cold allodynia in both wild type and nNOS (−/−) mice. These data suggest that nNOS expression in the sensory nervous system is not be major factor in neuropathic pain, other molecules such as GABA and calcium channel are more important in mechanical and cold allodynia than the nNOS expression in a mouse model of peripheral neuropathy.

In conclusion, nNOS expression in the sensory nervous system may be partially associated with development and/or maintenance of neuropathic pain behavior in a mouse model of peripheral neuropathy.

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Korean J Phys Anthropol
18(4): 303 ~ 312, 2005

# nNOS 결핍 생쥐에서의 신경병증성 통증의 변화

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**간추림** : 신경손상 후 척수나 후근신경절에서 NO 생성 변화가 이질통(allodynia)에 관여하는 것으로 알려져 있다. 반면 nNOS의 발현이 이질통의 유발과 유지에 관여하지 않는다는 보고도 있다. 본 연구에서는 감각신경계통의 nNOS의 발현이 신경병증성 통증에서의 역할을 규명하고자 하였다.

정상조군 및 nNOS 결핍 생쥐에서 말초신경 손상 후 von Frey 및 아세톤 테스트를 시행하였으며, 이들 쥐에 nNOS 억제제를 투여하여 통증의 변화를 확인하였다.

nNOS 결핍 생쥐에서는 말초신경 손상 후 정상대조군 생쥐보다 물리적 이질통이 약간 감소하였다. 정상대조군에서는 nNOS 억제제인 L-NAME 투여에 의해 물리적 이질통이 약간 감소하였다. 반면에, gabapentin은 정상대조군 및 nNOS 결핍 생쥐 모두에서 신경병증성 통증(물리적 및 냉 이질통)을 현저히 감소시켰다.

이상의 결과는 생쥐의 신경병증성 모델에서 감각신경계의 nNOS의 발현이 물리적 이질통의 유발과 유지에 일부 관여하고 있음을 보여준다.

**찾아보기 낱말** : nNOS, 신경병증성 통증, 이질통, nitric oxide

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