Animal Trial

Mode of Action of Amitriptyline Against Neuropathic Pain via Specific NF-kB Pathway Suppression

YouMi Hwang, MD, PhD[1,2] and So Young Kwon, MD, PhD[3]

From: [1]Department of Cardiology, St. Vincent's Hospital, Catholic University of Korea, Seoul, Republic of Korea; [2]Catholic Research Institute for Intractable Cardiovascular Disease (CRID), College of Medicine, Catholic University of Korea, Seoul, Republic of Korea; [3]Department of Anesthesiology and Pain Medicine, St. Vincent's Hospital, Catholic University of Korea, Seoul, Republic of Korea

Address Correspondence:
So Young Kwon, MD, PhD
Department of Anesthesiology and Pain Medicine, St. Vincent's Hospital, The Catholic University of Korea
Seoul 16247, Republic of Korea
E-mail: so-young@catholic.ac.kr

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Background: Neuropathic pain occurs for various reasons involving adenosine receptors. One of several drugs used to control neuropathic pain is amitriptyline, a tricyclic antidepressant. Amitriptyline has an antinociceptive effect on the A3 adenosine receptor (A3AR). However, the exact mechanisms underlying A3AR activation remain unclear.

Objectives: By investigating the effects of amitriptyline on neuropathic pain mitigation and its impact on inflammatory pathways via A3AR activation, we aimed to provide novel insights into the potential mechanisms of action of amitriptyline in neuropathic pain management. These insights could potentially revolutionize the way we understand and treat neuropathic pain.

Study Design: We used Sprague-Dawley rats for the neuropathic pain models. The rats were sorted into the sham (control), neuropathic pain (NP+NS), and neuropathic pain and amitriptyline (NP+AMI) groups. Each group consisted of 8 rats. This design allowed us to compare the effects of amitriptyline on neuropathic pain in a control group and a group that experienced neuropathic pain without amitriptyline treatment.

Setting: St. Vincent's hospital, research institute of medical science.

Methods: Normal saline and amitriptyline were injected intraperitoneally into rats using a subcutaneously implanted osmotic pump. A week after the procedure, nuclear factor kappa B (NF-κB) and a related proinflammatory cytokine (TNF-α) were quantified using immunoblotting or reverse-transcription PCR.

Results: Our results brought positive news. The NF-κB concentrations of the groups were not different from one another, indicating a stable baseline. The control and NP+NS groups showed relatively increased activation of the mu-opioid receptor (MOR) and proinflammatory cytokines, including TNF-α, but demonstrated no intergroup difference. However, the MOR and TNF-α concentrations were markedly lower in the NP+AMI group than in the control or NP+NS groups ($P = 0.02$, 0.002, respectively). This difference suggests a potential for amitriptyline to reduce inflammation. The paw withdrawal threshold test revealed a recovery response to mechanical allodynia for the NP+AMI group ($P < 0.05$), indicating a positive impact on neuropathic pain.

Limitations: The experiment involved only a few mice, so the results may not be generalizable.

Conclusions: The release of proinflammatory cytokines via NF-κB expression and subsequent inflammatory responses is significantly associated with the development of neuropathic pain. Our study reveals that AMI effectively suppresses NF-kB-related proinflammatory cytokines, offering a promising avenue for treating pain related to peripheral nerve injuries. These findings provide valuable insights into neuropathic pain management.

Key words: amitriptyline, neuropathic pain, NF-kB, TNF- α

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Neuropathic pain has various causes involving neuroinflammation and G-protein-coupled receptors. Adenosine regulates several physiological and pathological conditions in the heart and neurocardiac axis through G-protein-coupled adenosine receptors. The 4 major adenosine receptors are A1, A2A, A2B, and A3. The activation of A1 in cardiomyocytes attenuates the chronotropic and inotropic effects on the heart. In contrast, the anti-inflammatory, vasodilatory, and antiplatelet effects on the coronary and vascular systems are primarily mediated by A2A activation during ischemia. Under vascular damage and inflammatory conditions, A2B receptors are upregulated and exert cardioprotective effects. Injured peripheral nerves can cause nervous system abnormalities, and these neuropathies may progress to functional disabilities via A2 and A3 activation caused by ischemia and neuroinflammation. Adenosine receptors have several overlapping neuroprotective and anti-inflammatory actions, but their roles cannot be substituted, and the A3A receptor is overexpressed in inflammatory and cancer cells (1-3).

Amitriptyline is a widely used tricyclic antidepressant (TCA) known for its effectiveness in treating neuropathic pain. Its mechanisms of action include blocking the reuptake of serotonin and norepinephrine, leading to increased synaptic concentrations of these neurotransmitters. Additionally, amitriptyline acts as an antagonist of NMDA receptors, reducing neuronal activation related to nerve tissue damage and harmful mechanical stimuli. Notably, amitriptyline has been found to have an antinociceptive effect on the A3 adenosine receptor (A3AR), which plays a significant role in modulating inflammatory responses and pain pathways. The exact mechanisms underlying amitriptyline's activation of the A3AR remain unclear (4). Adenosine receptor activation during neuropathic pain produces adenosine monophosphate-activated kinase (AMPK), which mitigates neuropathic pain in mammals by targeting rapamycin (MTOR) or mitogen-activated protein kinase (MAPK) signaling (5-8).

The number of patients with neuropathic pain is increasing, and the types of neuropathic pain are diverse. They include malignancy-related pain, diabetic polyneuropathy, postsurgical neuropathic pain, and complex regional pain syndrome (CRPS) (9). Neuropathic pain is frequently intractable or difficult to treat and reduces the quality of life, leading to psychological, physical, and social dysfunction in patients (9-11). The pathophysiology of neuropathic pain has yet to be elucidated. However, cytokines and neurotransmitters increase at the spine level, where nerve damage occurs. The increase in cytokines and neurotransmitters activates pain-related receptors, including adenosine receptors, and microglial responses following pro-inflammatory responses to nerve damage (7,12,13).

This study evaluated how amitriptyline reduced neuroinflammation in neuropathic pain by activating A3AR. This study also assesses the effect of amitriptyline against neuropathic pain and its impact on pain-related inflammatory responses.

Methods

Study Design and Setting

This animal study was conducted with permission from the university's ethics committee (IRB 14-06) based on the Institutional Laboratory Animal Care Committee. Male Sprague-Dawley rats were used for the neuropathic pain models. We used the Kim and Chung method (14) to prepare and analyze the rodent models of neuropathic pain. The rats used for the experimental models weighed 180–200 g.

Procedures

Pentobarbital sodium (50 mg/kg) was injected intraperitoneally to induce anesthesia. A paramedian linear incision was made from the level of the third lumbar vertebra to the second sacral vertebra, and the transverse process of the lumbar vertebra was removed to expose the spinal nerves. The exposed nerves were sutured with 5-0 silk for nerve damage, and the surgical wound was sutured for closure. A day after the surgical procedure, a subcutaneous osmotic pump was implanted.

Between the first and seventh days after surgical preparation, the paw withdrawal threshold test was performed using von Frey filaments (0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 10. 0, 15.0 g; Stoelting Co.). The paw withdrawal test was used to measure the number of paw withdrawal reactions when the von Frey filament was applied as a mechanical stimulus to the sole of the hind paw of the operated rat 10 times at 2–3-second intervals. Rats that showed withdrawal responses more than 5 times over 10 stimulations were considered successful neuropathic pain rat models (15). The control group underwent the same procedure without having their nerves tied. Rats that showed no paw mobility or failed to show mechanical allodynia after the procedure were excluded from the experiment. The effect of amitriptyline on neuropathic pain

was analyzed in the following 3 groups of rat models: (a) a group that received surgically induced neuropathic and amitriptyline (NP + AMI, n = 8; AMI was infused via subcutaneous osmotic pump [model 2ML1; Durect Corp.] with a speed of 15 µL/hour for 7 days), (b) a group that received surgically induced neuropathic pain and normal saline (NP + NS, n = 8; instead of AMI, normal saline was infused during the experiment), and (c) a sham group (control, n = 8; performed the same procedure without tying nerves). The withdrawal paw threshold test to mechanical stimuli was performed every day from the first to the seventh following the osmotic pump implant immediately after the surgical procedure).

On the eighth day after the surgical procedure, anesthesia with oxygen and 5% sevoflurane was administered to place the rats under deep sedation, at which point they were decapitated and their brains harvested. The spinal cords were also harvested after the rats' sacra and third lumbar vertebrae were cut simultaneously, as in the previously reported method (18,19). The brain and spinal cords were immediately frozen in liquid nitrogen and stored at -80°C. Changes in the NF-κB and µ-opioid receptors (MOR) were detected using immunoblotting. The expression of the NF-κB and tumor necrosis factor α (TNF-α) mRNAs were assessed using RT-PCR (16,17). Double-blinded researchers performed all experiments and analyses in the 3 experimental groups.

The refrigerated dorsal root ganglia and spinal cords of the rats were dissected on ice. The cells were immediately immersed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100; pH 7.4). After homogenization, the tissue was ultracentrifuged at 32,500 rpm and 4°C under vacuum conditions for one hour, and the supernatant was attained. The protein concentration in the supernatant was measured using the Bradford assay (Bio-Rad Laboratories), with bovine serum albumin used as the standard. Proteins were denatured by mixing with the Laemmli buffer.

For Western blot analysis, each sample containing 50 µg of protein was electrophoresed for 4 hours at 80 V on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Following electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at 20 V and 4°C overnight.

Specific primary antibodies detected NF-κB, TNF-α, and µ-opioid receptors. After being incubated with the primary antibody, the membrane was washed and incubated with the secondary antibody (goat anti-rabbit or anti-mouse IgG) for an hour. Bound antibodies were detected using electrochemiluminescence (ECL) reagents (Amersham Biosciences Corp.) and visualized using a Bio-Imaging System (UVP LLC).

To analyze mRNAs and TNF-α expression, harvested tissues were initially homogenized in 1 mL of Trizol reagent and allowed to stand at room temperature for 5 minutes to facilitate complete dissociation of nucleoprotein complexes. Next, 0.2 mL of 1-bromo-3-chloropropane was added, followed by centrifugation at 14,000 g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube, and an equal volume of phenol: chloroform (5:1, pH 4.7) (Sigma-Aldrich®) was added before centrifugation at 14,000 g for 10 minutes at 4°C to purify the RNA further. RNA was precipitated by adding 100% ethanol to the aqueous phase and incubating the mixture at -20°C for one hour, followed by centrifugation at 14,000 g for 10 minutes at 4°C. After the supernatant was discarded, the RNA pellet was washed with 75% ethanol, air-dried, and resuspended in diethylpyrocarbonate (DEPC)-treated water. The RNA was incubated for 5 minutes at 60°C to dissolve the pellet fully and then immediately cooled on ice. The concentration of RNA was determined by measuring the absorbance at 260 nm using a spectrophotometer. Reverse transcription was performed with 0.5 µg of the extracted RNA using reverse transcriptase (Promega) from the avian myeloblastosis virus (AMV) to synthesize cDNA in a volume of 50 µL.

For the PCR, a 10-µl reaction mixture was prepared. The mixture included dNTPs (each at 2.5 mM), one unit of I Start Taq DNA polymerase (Intron Biotechnology), and 20 µM of each primer, with 2 µL of synthesized cDNA as a template. PCR amplification was carried out for 35 cycles, each consisting of 30 seconds at 95°C for denaturation, 30 seconds at 55°C for annealing, and one minute at 72°C for extension, followed by a final extension step of 10 minutes at 72°C. β-actin (228 bp) was the internal control for successful mRNA extraction. The PCR products were separated by electrophoresis on an ethidium bromide-stained 2.0% agarose gel. The bands were visualized and quantified using the Gel Doc™ XR system (Bio-Rad Laboratories) (Table 1).

Statistics

All values are presented as mean ± SD. All statistical analyses were performed using IBM SPSS Statistics 28 (IBM Corporation). The mechanical withdrawal thresholds of the groups were compared using Student's t-test. One-way ANOVA was used to compare

the Western blot and RT-PCR results for the 3 groups, and Tukey's post-hoc test was used if a significant difference was found. *P*-values lower than 0.05 denoted statistical significance.

Results

Among the neuropathic rat models, the NP + AMI group showed a remarkable recovery of the withdrawal threshold after the fourth day of testing. However, the NP + NS group maintained this threshold. Western blot analysis of the mRNA expressions of TNF-α and MOR revealed no statistical differences between the control and NP + NS groups. Nonetheless, there were substantial reductions of the TNF-α and MOR expressions in the NP+AMI group (Fig. 1). RT-PCR analysis of TNF-α in the spinal nerves showed a marked increase in mRNA expression of TNF-α in the NP + NS and control groups ($P = 0.008, 0.006$, respectively). In contrast, the NP + AMI group showed a significant decrease in the mRNA expression of MOR ($P = 0.02, 0.002$, respectively). There were no statistical differences in the concentrations of the signaling protein (NF-κB) of the NP + AMI group and the other 2 groups (Fig. 2).

Discussion

This study confirmed that the concentration of TNF-α increased markedly in a rodent neuropathic pain model. The use of amitriptyline in neuropathic rats notably decreased MOR and related pro-inflammatory responses; however, NF-κB activation was similar in all groups, suggesting the amitriptyline action was related to more specific NF-κB-related inflammatory pathway suppression (Fig. 3).

The development of neuropathic pain involves diverse pathophysiological mechanisms in the peripheral and central nervous systems (9,18). The pathophysiology of neuropathic pain involves the myriad processes of spinal dorsal horn desensitization, which leads to augmented excitability in the processing of the nociceptive afferent nerve pathways (13,19-23).

Table 1. *RT-PCR primer sequences.*

Primer	Accession number	Sequence (5' → 3')	Size (bp)	Cycle number	Annealing temperature (°C)
β-actin	NM031144	F: AGCCATGTACGTAGCCATCC r) CTCTCAGCTGTGGTGGTGAA	228	35	55
TNF-α	X66539 S40199	F: TACTGAACTTCGGGGTGATCGGTCC r) CAGCCTTGTCCCTTGAAGAGAACC	295	35	62

RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor α.

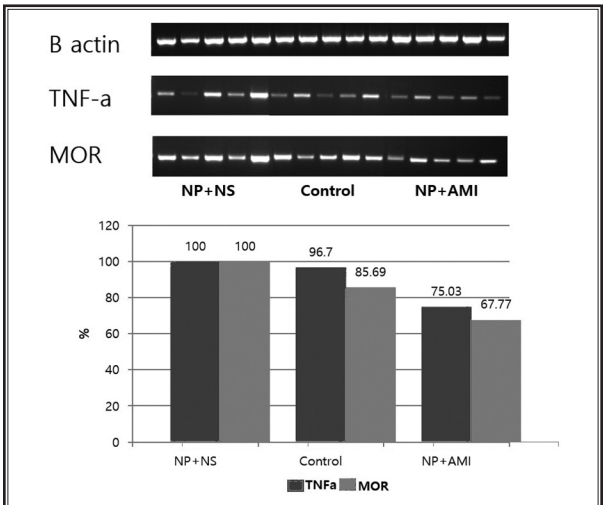

Fig. 1. *Western blot of TNF-a and MOR and bar graph comparing the TNF-a and MOR ratio among the study groups.*
TNF-a, tumor necrosis factor a; MOR, mu-opiod receptor

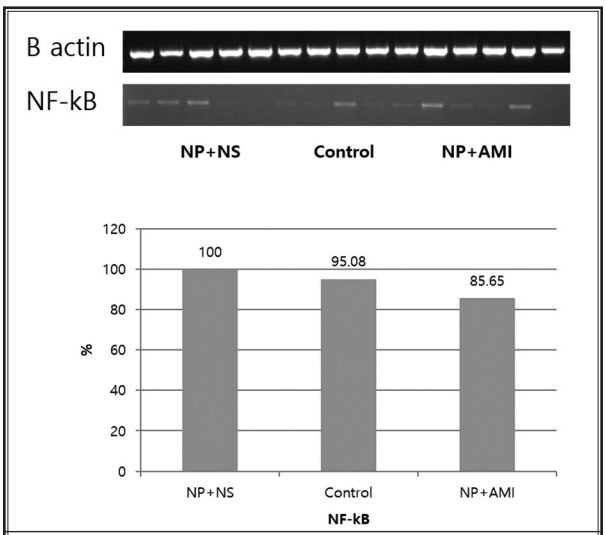

Fig. 2. *Western blot of NF-Kb and bar graph comparing the NF-Kb among the study groups.*
NF-Kb, nuclear factor Kappa B

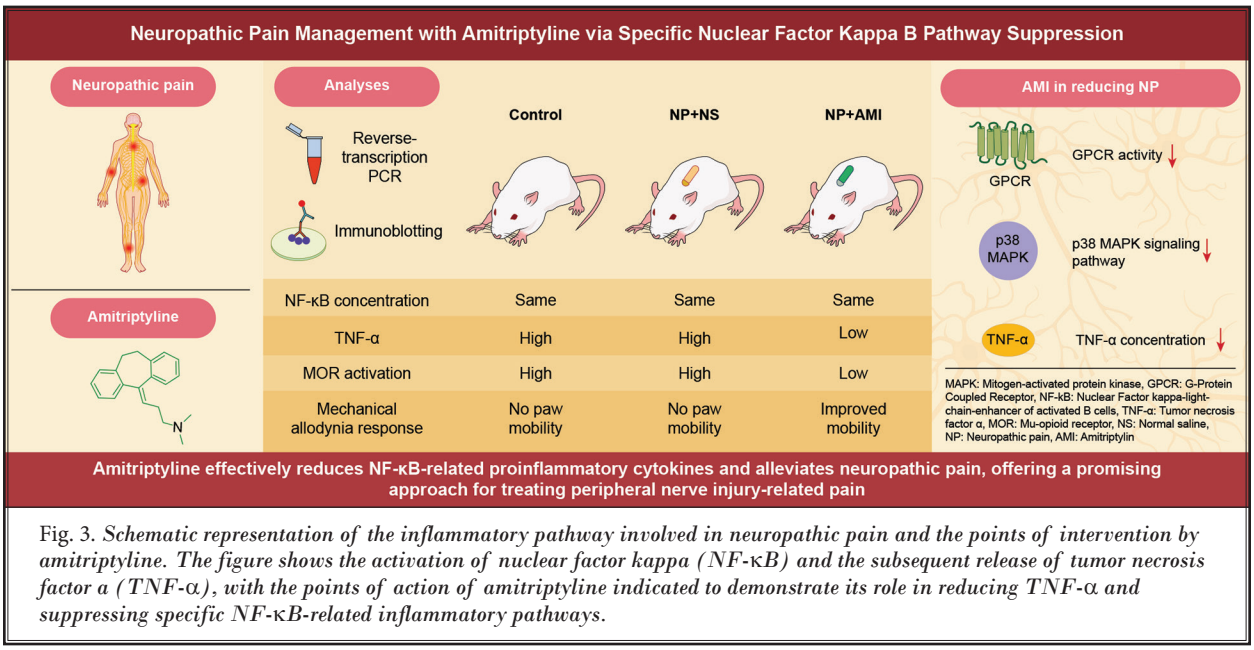

Fig. 3. *Schematic representation of the inflammatory pathway involved in neuropathic pain and the points of intervention by amitriptyline. The figure shows the activation of nuclear factor kappa (NF-κB) and the subsequent release of tumor necrosis factor α (TNF-α), with the points of action of amitriptyline indicated to demonstrate its role in reducing TNF-α and suppressing specific NF-κB-related inflammatory pathways.*

Tissue damage, inflammation, or nerve injury results in chronic neuropathic pain characterized by hyperalgesia and allodynia (20,22,24-27). Excitatory amino acids (EAA) are essential for the ischemic cascade during neuropathic pain, and the elevation of EAA concentrations leads to MAPKs, including ERK 1/2, JNK, and p38 processes (17,28), which eventually can cause cell death (1,4,19). During this process, the number of glutamate receptors at neural junctions increases, thus in turn increasing those junctions' interactions with glutamate (13). While an activated metabotropic receptor opens the ion channel, it must be accompanied by several neuropathic elements (cAMP, cGMP, IP3, and DAG) (29). Various GPCR-related receptors influence glutamate transporters, including phosphatidylinositol 3-kinase, MAPKs, and CREB, which affect protein kinase C (PKC) (30). Increased PKC and NMDA concentrations are essential during the pathogenesis of neuropathic pain (31).

Spinal sigma-1 receptor activation causes mechanical allodynia with the phosphorylation of p38 MAPK through the transient receptor potential A1 (32). The phosphorylation of NO-mediated protein kinases and PKC enhances receptors in the cell membrane and Ca2+ pathways. PKC and NO-mediated protein kinases also decrease the efficiency of the μ-opioid receptor (MOR) and interfere with the coupling of the MOR and G proteins, which may explain the enhanced tolerance to the analgesic effect of morphine.

This study aimed to elucidate the effects of amitriptyline, an NMDA antagonist, in a rodent model of neuropathic pain. Amitriptyline weakens harmful stimuli as a noncompetitive antagonist of EAAs and suppresses allodynia and hyperalgesia in various neuropathic diseases. Classified as a TCA, amitriptyline not only acts as an NMDA receptor antagonist but also inhibits the reuptake of both serotonin and norepinephrine neurotransmitters. This dual reuptake inhibition enhances synaptic concentrations of these neurotransmitters, contributing to amitriptyline's analgesic and mood-stabilizing effects. Additionally, amitriptyline's anticholinergic properties and modulation of ion channels further contribute to this antidepressant's multifaceted pharmacological profile.

Other studies have described the effects of amitriptyline on pain control. Some researchers have reported that amitriptyline is concentration-dependent, reverses the conductance of Na+ channels in human neurons, and changes the voltage-dependent activities of various ion channels. The present study was performed with the expectation that amitriptyline-mediated reduction in p38 MAPKs was associated with cell signaling of adenosine receptors and inflammation reduction. Adequate pain control is possible through the repetitive use of sufficient doses of amitriptyline to suppress NMDA activation at the spinal-cord level.

However, in addition to its primary actions, amitriptyline exhibits several pharmacological effects, including anticholinergic and antihistaminic activities, which can lead to adverse results such as dry mouth,

constipation, urinary retention, and sedation. Due to amitriptyline's potential for cardiotoxicity, it is contraindicated in patients with certain cardiac conditions, such as recent myocardial infarction, arrhythmias, and prolonged QT intervals. Furthermore, amitriptyline should not be used concurrently with monoamine oxidase inhibitors (MAOIs) due to the risk of severe interactions, including serotonin syndrome.

Limitations

This study had several limitations. The results are difficult to generalize because the study involved only a few mice. Said results are nonetheless meaningful, since statistically significant differences were observed among the groups. Another limitation of this study is that the differences in pain threshold or degree of recovery that are inevitable between individuals who receive the same surgical manipulations could not be determined; however, this limitation is considered difficult to correct in animal experiments.

Conclusions

We found a notable increase in intracellular signaling proteins and TNF-α in rat models of neuropathic pain. The treatment of neuropathic pain with amitriptyline suppressed GPCR activity and decreased the p38 MAPKs signaling protein production. This study provides substantial evidence supporting the effectiveness of TCAs for patients with chronic peripheral neuropathy. Furthermore, TCAs may also be effective against other neuropathic conditions, such as malignancy-related pain, spinal pain, and various neuropathic pains, including CRPS. This study aimed to highlight that TCAs were a versatile form of therapy for managing neuropathic pain, contributing to the optimization of pain management strategies for these conditions. Future studies on adenosine should explore its mechanisms of action and therapeutic potential in various contexts. Doing so will encourage innovative studies to uncover new aspects and applications of adenosine in science and medicine.

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