

Original Article

Aloin attenuates NMDA receptor by modulating calcium response on substantia gelatinosa neurons of the trigeminal subnucleus caudalis in mice

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ABSTRACT Aloe vera extracts, particularly aloin, have been widely used in traditional Chinese medicine for their anti-inflammatory and detoxifying properties. Recent studies suggest that aloin may also exert neuroprotective effects, though its specific mechanisms remain unclear. Glutamate excitotoxicity, a critical factor in neurodegenerative and pain-related disorders, is mediated primarily by N-methyl-D-aspartic acid (NMDA) receptors, which regulate calcium influx and neuronal excitability. Meanwhile, substantia gelatinosa (SG) of the trigeminal subnucleus caudalis (Vc) plays a central role in processing orofacial pain, where glutamate is a key excitatory neurotransmitter. Using whole-cell patch-clamp electrophysiology and calcium imaging techniques in mice SG neurons of the Vc, we examined aloin's effects on glutamate receptor-mediated responses. Aloin selectively inhibited NMDA-induced responses without affecting AMPA or kainate receptor activity. The suppression of NMDA-mediated currents by aloin was linked to changes in Ca^{2+} influx but occurred independently of voltage-gated sodium channels and action potential generation. Calcium imaging revealed that aloin reduced NMDA-induced intracellular calcium influx, supporting its role in mitigating NMDA-mediated excitotoxicity. Furthermore, aloin significantly suppressed the spontaneous firing activity of SG neurons, suggesting its broader regulatory effects on neuronal excitability. Our findings demonstrate that aloin attenuates NMDA receptor-induced excitatory signaling by regulating calcium response. This specificity highlights aloin's potential as a therapeutic candidate for conditions involving glutamate excitotoxicity and orofacial pain regulation.

INTRODUCTION

Pain signals from the orofacial region are transmitted via the trigeminal pathway, beginning with nociceptors in the peripheral tissues. These nociceptors detect painful stimuli and send signals through A δ and C fibers, which travel along the trigeminal nerve (cranial nerve V) to the trigeminal ganglion [1]. From there, the

signals enter the brainstem and synapse in the trigeminal spinal nucleus, particularly in the caudal portion (trigeminal subnucleus caudalis, Vc). Within this region, the substantia gelatinosa (lamina II) (SG) neuron plays a crucial role in modulating pain signals [2].

Glutamate serves as the principal excitatory neurotransmitter in the central nervous system (CNS), mediating fast synaptic transmission and synaptic plasticity essential for learning and



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memory [3]. However, excessive or prolonged activation of glutamate receptors – especially ionotropic types such as *N*-methyl-D-aspartic acid (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors – leads to neuronal hyperexcitability, characterized by sustained depolarization, calcium overload, and activation of cytotoxic signaling cascades [4,5]. This phenomenon, known as glutamate-induced excitotoxicity, contributes to neuronal injury and underlies various neuropathological and pain-related conditions [5,6].

Aloin, a bioactive compound found in aloe vera, is a naturally occurring anthraquinone glycoside known for its diverse pharmacological and industrial applications [7]. Aloin has demonstrated notable anti-nociceptive effects in preclinical studies, making it a promising candidate for pain management. Aloin exhibits analgesic and anti-inflammatory activity primarily through inhibition of inducible nitric oxide synthase and suppression of pro-inflammatory cytokine release, rather than by directly blocking cyclooxygenase-2 (COX-2) or prostaglandin synthesis pathways [8]. These mechanisms contribute to its efficacy in alleviating both inflammatory and neuropathic pain in animal models [6]. In addition to its anti-nociceptive properties, aloin also offers neuroprotective benefits. Its antioxidant and anti-inflammatory actions help reduce oxidative stress and neuroinflammation – two major drivers of neurodegenerative diseases such as Alzheimer's and Parkinson's [9]. Aloin has been reported to decrease neuronal apoptosis, support mitochondrial function, and suppress the production of pro-inflammatory cytokines [10]. Moreover, its ability to modulate glutamate excitotoxicity and maintain calcium homeostasis further supports its potential in preventing neuronal damage and degeneration [11].

The effect of aloin within the trigeminal pathway, particularly its interaction with SG neurons, have not been previously investigated. Therefore, this study aimed to investigate the effects of aloin on SG neurons in the Vc and explore its potential mechanisms against glutamate-induced excitotoxicity. To test whether aloin mitigates excitotoxic damage, we assessed its impact on ionotropic glutamate receptor (iGluR)-mediated excitatory synaptic activity in SG neurons from juvenile mice using patch-clamp electrophysiology. This approach allowed for a detailed assessment of aloin's impact on synaptic transmission and neuronal excitability, providing insights into its potential therapeutic role in modulating trigeminal pain processing and preventing glutamate-mediated neuronal dysfunction.

METHODS

Brainstem slice preparation

Immature Institute of Cancer Research (ICR) mice (8–20 postnatal days, both male and female) were kept in a controlled environment with the following conditions: free access to food

(DBL) and water, 12-h light/dark cycle with lights on at 6 AM. Experiments were conducted with permission from the Institutional Animal Care and Use Committee of Jeonbuk National University (JBNU NON2023-065) and were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals.

The brainstem slice preparation followed the protocol outlined in our previous study [12]. Mice were euthanized and their brains were rapidly dissected and immersed in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF, prepared with ultrapure water from Water Purification Systems, Dream Mega (MDM Co., Ltd.), contained (in mM): 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.2 MgCl₂, 11 D-glucose, 25 NaHCO₃, and 1.4 NaH₂PO₄ (pH 7.3–7.4, oxygenated with 95% O₂ and 5% CO₂). The brainstem, including the Vc, was secured to 4% agar using cyanoacrylate and sectioned in ice-cold ACSF with a vibratome (VT1200S; Leica Biosystems). Coronal slices (180–200 μ m in thickness) containing the rostral part of the Vc were then incubated in oxygenated ACSF at room temperature for at least one hour before electrophysiological recordings.

Whole-cell patch-clamp recording

Brain slices were examined using an upright microscope (BX51WI; Olympus) and Nomarski differential interference contrast optics. Coronal slices were placed in the recording chamber, where they were fully submerged and constantly perfused with oxygenated ACSF at a flow rate of 4–5 ml/min. SG neurons in the Vc were identified as a translucent band situated medially to the spinal trigeminal tract and along the lateral edge of the brain slices. For electrophysiological recordings, SG neurons were randomly selected from these regions.

Patch pipettes were fabricated by pulling thin-walled borosilicate glass capillary tubing (PG52151-4; WPI) using a Flaming Brown puller (P-97; Sutter Instruments Co.). They were then filled with a high-chloride solution (in mM: 1 MgCl₂, 140 KCl, 1 CaCl₂, 10 HEPES, 10 EGTA, 4 MgATP, pH = 7.3 with KOH), which enhanced inhibitory postsynaptic current (IPSC) detectability for monitoring spontaneous activity. For glutamate-induced responses, a K⁺-based pipette solution was used to preserve physiological ionic conditions. Although IPSCs or mGluR-related K⁺ conductance cannot be entirely excluded, our brief agonist applications were designed to isolate fast iGluR-mediated inward currents. Spontaneous action current firing was recorded using the cell-attached configuration in voltage-clamp mode with a holding potential of 0 mV.

When filled with a KCl-based internal solution, the recording electrode tip resistance ranged from 4 to 7 M Ω . Once giga-seal with SG neurons was created, a slight negative pressure was applied to achieve the whole-cell configuration. Signals were amplified using an Axopatch 200B (Molecular Devices), filtered at 1 kHz with a Bessel filter, and digitized at a sampling rate of 1 kHz. Membrane current changes were recorded *via* a Digidata

1440A interface (Molecular Devices) transmitted to a computer. All experiments were conducted at room temperature. Data was acquired and analysed using Clampex 10.6 (Molecular Devices).

Standard ACSF was used to record AMPA- and kainate-induced currents, whereas NMDA-induced currents were recorded in ACSF continuously supplemented with glycine (1 μ M) to enhance NMDA receptor (NMDAR) activity and reduce desensitization [13]. Some recordings were performed in Na^+ -free external solution by replacing equimolar NaCl with choline chloride to reduce passive Na^+ influx [14]. Additionally, calcium-free ACSF solution was also prepared with the following composition (in mM): 126 NaCl, 2.5 KCl, 3.6 MgCl_2 , 1.4 NaH_2PO_4 , 11 D-glucose, 25 NaHCO_3 , and 2 EGTA.

To ensure objectivity, researchers remained unaware of the experimental groups throughout different phases of the study. Additionally, all experiments were performed at the same time of day to minimize the potential effects of circadian rhythms on neuronal nociception.

Fluorescence Ca^{2+} imaging

To visualize calcium ion uptake in neurons, the Fluo-3 AM staining assay was conducted. A 1 mM stock solution of Fluo-3 AM (F1241, ThermoFisher) was prepared by dissolving the dye in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Brain slices were incubated in oxygenated ACSF containing 1 μ M Fluo-3 AM for 40 min in darkness. To preserve fluorescence, all preparation and loading procedures were conducted at room temperature using foil-wrapped tubes. Following incubation, the slices were rinsed three times with ACSF and allowed to rest for an additional 30 min before imaging. Once fluorescence signals stabilized, the experiment was performed using a sequential application protocol: NMDA (5 min), NMDA with aloin (5 min), washout (5 min). Fluorescent images were captured at one-minute intervals using a fluorescence microscope (EVOS M5000; ThermoFisher). To assess spontaneous Ca^{2+} signal variations in response to aloin, regions corresponding to laminae I-II were selected within the field of view, and mean pixel intensity (MPI) was analyzed for each frame using EVOS M5000 software. Fluorescence intensity data were scaled to a relative value ($\Delta F/F_0$, where $\Delta F = F - F_0$), with F indicating the average fluorescence intensity for each frame and F_0 representing the baseline average intensity.

Chemicals

The following chemicals were prepared before experiments: Tetrodotoxin (TTX) and AMPA (Tocris Bioscience), Fluo-3 AM (ThermoFisher), aloin, glycine, glutamate, NMDA, kainate and ACSF components (Sigma-Aldrich). The stock concentration of aloin was 200 mM, and the final DMSO concentration in the solution remained below 0.3%, ensuring no effect on the membrane potential of SG neurons.

Data and statistics analysis

Mini-Analysis software (ver. 6.0.7; Synaptosoft Inc.) was used to analyze neuronal firing, with the control, aloin application, and washout phases each analyzed for a fixed 2-minute period. EVOS M5000 software was used for calcium signal analysis. The relative percentage was determined by dividing the target response by the control response and then multiplying by 100. Data was presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using one-way ANOVA, with significance levels indicated as ns (not significant), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). The value "n" in the figures represents the number of SG neurons used. Origin 8 software (Origin-Lab Corp) was used to perform statistical analysis.

RESULTS

The effects of aloin on SG neurons were evaluated by cell-attached and whole-cell voltage-clamp recordings on 60 SG neurons in the Vc from 42 juvenile mice. Notably, aloin did not induce any significant changes in the holding current required to maintain neurons at -60 mV.

Effect of aloin on the spontaneous firing activities of SG neurons of the Vc

To evaluate the effect of aloin on the spontaneous firing activity of SG neurons, recordings were performed in cell-attached voltage-clamp mode. Perfusion with 500 μ M aloin for 3 min significantly reduced spontaneous firing activity, which recovered after 10 min of washout (Fig. 1A). Specifically, the firing frequency of SG neurons was significantly reduced to $60.6 \pm 4.2\%$ in the presence of aloin and subsequently recovered to $90.5 \pm 5.6\%$ relative to the control group (one-way ANOVA followed by Tukey's *post-hoc* test, $n = 9$, $p < 0.0001$, $F_{1,8,14,3} = 31.7$; Fig. 1B). These results indicate that aloin exerts a suppressive effect on the excitability of

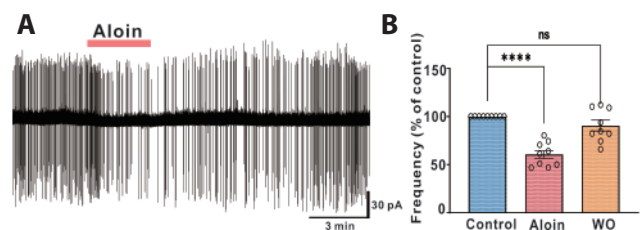


Fig. 1. Aloin suppressed the spontaneous firing activities of substantia gelatinosa (SG) neurons. (A) A representative trace of action currents recorded from a SG neuron in the application of aloin (500 μ M) in the cell-attached mode. (B) A bar graph showing the firing frequency percentages in the presence and washout of aloin, $n = 9$, **** $p < 0.0001$, one-way ANOVA. Data are represented as mean $\pm$ SEM. Horizontal bars indicate the duration of drug application. ns, not significant.

SG neurons in the Vc region.

Inhibitory effect of aloin on glutamate-mediated activation in SG neurons of the Vc

Given glutamate's role as a ubiquitous excitatory neurotransmitter in the CNS, acting through ligand-gated ion channels, we investigated the modulatory effect of aloin on glutamate-induced responses and its subtype agonists. Whole-cell voltage-clamp recordings revealed that the exogenous application of glutamate (100 μ M, 2 min) induced inward currents with a mean amplitude of 96.2 ± 15.4 pA ($n = 15$; Fig. 2A). Continuous perfusion with aloin (500 μ M), initiated 3 min before and maintained during glutamate application, significantly attenuated glutamate-mediated responses, reducing the current amplitude to 55.1 ± 9.8 pA ($n = 15$; Fig. 2B). This inhibitory effect of aloin was reversible, as the current amplitude recovered to approximately 90% of the control value after a ten-minute washout (one-way ANOVA followed by Tukey's *post-hoc* test, $n = 15$, $p = 0.0002$, $F_{1,4,19.2} = 16.9$).

To further elucidate whether aloin directly modulates iGluRs in SG neurons or if its effects are involved in action potential-dependent mechanisms, we examined its impact on glutamate responses in the presence of TTX (0.5 μ M), a voltage-gated sodium channel blocker. Under these conditions, aloin (500 μ M) consistently inhibited glutamate-induced currents (TTX + glutamate: 83.4 ± 12.1 pA; TTX + aloin: 47.6 ± 8.9 pA; one-way ANOVA followed by Tukey's *post-hoc* test, $n = 12$, $p = 0.001$, $F_{1,4,15.2} = 13.3$; Fig. 2C, D). These findings suggest that aloin exerts a direct inhibitory effect on iGluRs, independent of action potential generation.

Alain selectively inhibits NMDAR-mediated responses in SG neurons

To further characterize the inhibitory effects of aloin on iGluRs in SG neurons of the Vc, we examined its influence on responses mediated by specific glutamate receptor agonists, including AMPA, NMDA, and kainate. In control conditions, each agonist was applied for 2 min following a five-minute baseline recording. To assess the impact of aloin, the compound was perfused for 3 min before agonist application, and the agonists were subsequently introduced into the ACSF without removing aloin.

In contrast to NMDARs, AMPA receptors (AMPA) and kainate receptors do not depend on extracellular Mg^{2+} blockade or the presence of co-agonists like glycine or serine for their activation and channel opening. Therefore, we hypothesized that aloin would not significantly affect AMPAR- or KAR-mediated responses. To test this hypothesis, AMPA and kainate were applied in the absence and presence of aloin. In all tested neurons, bath application of AMPA (3 μ M) induced robust inward currents with a mean amplitude of 103 ± 27.0 pA ($n = 7$). Pretreatment with aloin (500 μ M) for 3 min did not significantly alter AMPA-mediated responses (aloin + AMPA: 97.2 ± 27.4 pA, $n = 7$, $p = 0.057$, $F_{1,6,9.6} = 4.1$; Fig. 3A, B). Similarly, 500 μ M aloin did not significantly affect the responses induced by kainate (10 μ M) (kainate: 71.3 ± 7.6 pA; aloin + kainate: 67.3 ± 8.2 pA; one-way ANOVA followed by Tukey's *post-hoc* test, $n = 8$, $p = 0.05$, $F_{1,7,11.6} = 4.1$; Fig. 3C, D).

Fig. 4A shows a distinct inward current elicited by NMDA exposure (30 μ M), indicating NMDAR activation in the presence of extracellular Ca^{2+} . To ensure full receptor activation and reduce desensitization, glycine (1 μ M) was co-applied as an essential co-agonist for NMDARs during these experiments. Notably, aloin

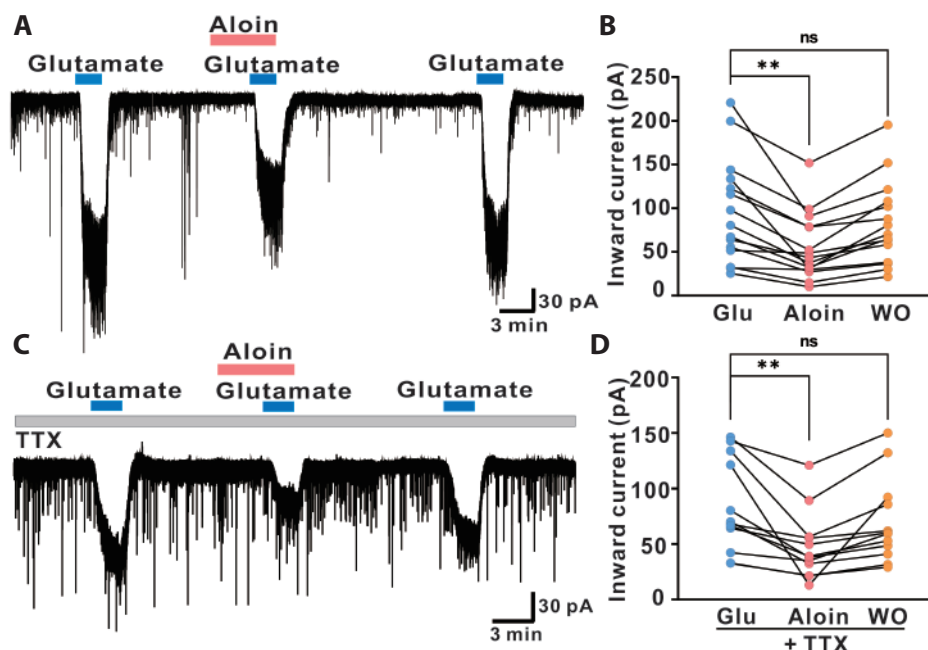


Fig. 2. Effects of aloin on glutamate-mediated responses in substantia gelatinosa neurons. (A) Representative traces showing inhibition of glutamate-mediated responses by aloin (500 μ M) in the presence of glutamate alone (100 μ M). (B) Line series plots showing inward currents induced by glutamate in the absence and presence of aloin, $n = 15$. (C) Representative traces showing inhibition of glutamate-mediated responses by aloin (500 μ M) in the presence of tetrodotoxin (TTX, 0.5 μ M), a voltage-sensitive sodium channel blocker. (D) Line series plots showing inward currents induced by glutamate co-application of TTX in the absence and presence of aloin, $n = 12$, $**p < 0.01$, one-way ANOVA. ns, not significant.

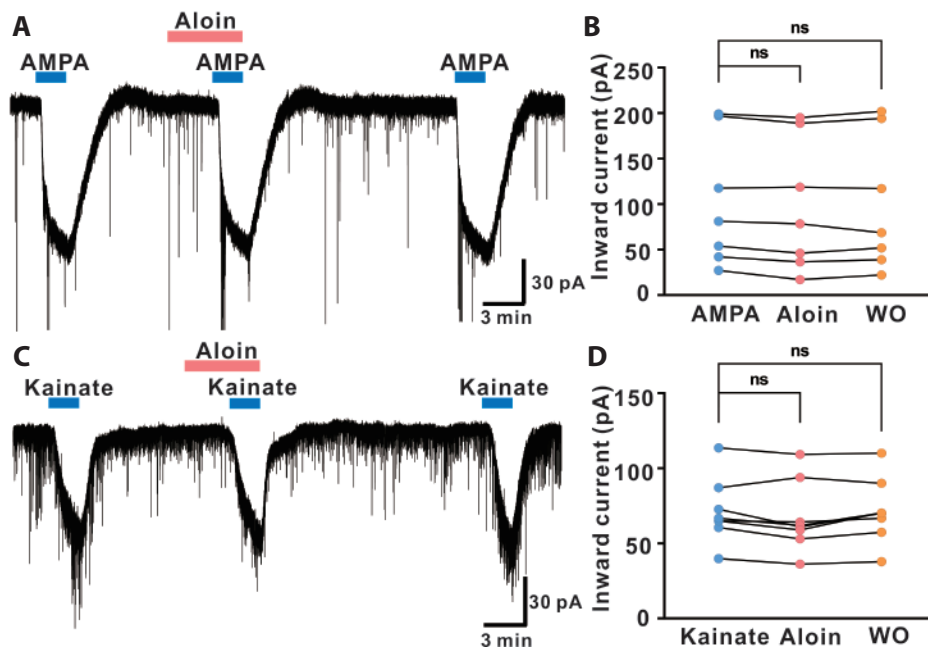


Fig. 3. Effects of aloin on α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and kainate-mediated responses. (A, C) Representative traces showing inward currents induced by AMPA (3 μ M) and Kainate (10 μ M) in the absence and presence of aloin (500 μ M). (B, D) Line series plots showing inward currents induced by AMPA ($n = 7$) and Kainate ($n = 8$) in the presence of aloin and after washout of aloin compared to AMPA and Kainate alone, one-way ANOVA. ns, not significant.

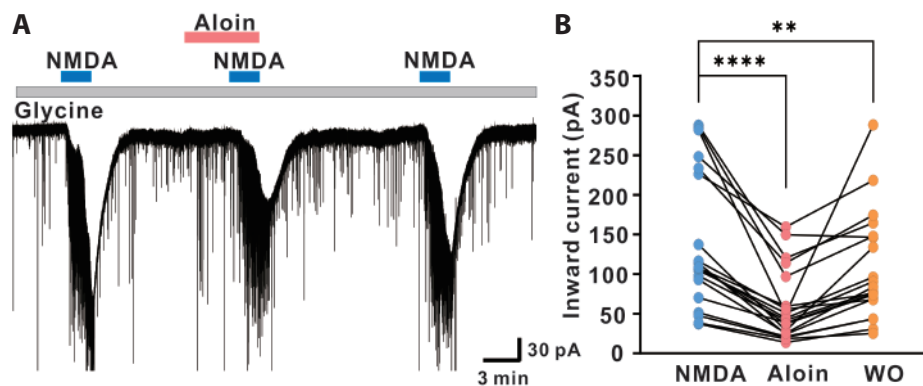


Fig. 4. Effects of aloin on N -methyl-D-aspartic acid (NMDA)-mediated responses. (A) A representative trace showing inward currents induced by NMDA (30 μ M) in the absence and presence of aloin (500 μ M). (B) A line series plot showing inward currents induced by NMDA in the presence of aloin and after washout of aloin compared to NMDA alone, $n = 20$, ** $p < 0.01$, **** $p < 0.0001$, one-way ANOVA.

(500 μ M) rapidly and reversibly suppressed NMDA-induced activity in SG neurons (alone: 140 ± 19.4 pA; aloin: 58.5 ± 10.0 pA; washout: 106 ± 15.0 pA; one-way ANOVA followed by Tukey's *post-hoc* test, $n = 20$; $p < 0.0001$, $F_{1.8, 33.8} = 26.1$; Fig. 4B). Additionally, no significant sex-based difference was observed in aloin-mediated NMDA current suppression, with relative inhibition of $40.9 \pm 5.9\%$ in males ($n = 9$) and $42.6 \pm 2.9\%$ in females ($n = 11$), compared to NMDA alone. Collectively, these results highlight the inhibitory effect of aloin on NMDAR-mediated responses in SG neurons of the Vc.

Aloin's effects on the calcium ion influx induced by NMDARs

The mechanism by which aloin modulates NMDAR-mediated responses in SG neurons was then investigated. Previous studies have shown that the initial rapid influx of extracellular Ca^{2+} , followed by delayed Ca^{2+} dysregulation, plays a key role in glutamate-induced excitotoxicity in certain neuronal cultures

[15,16]. Therefore, in this study, we investigated the effect of aloin on NMDA-mediated responses under calcium-free conditions. Typically, in the absence of extracellular Ca^{2+} , NMDA application resulted in a significantly reduced mean amplitude of inward currents compared to those recorded in standard ACSF ($18.1 \pm 3.7\%$ of the control; $n = 10$; Fig. 5). This finding suggests that the majority of the current generated through NMDAR activation in SG neurons is largely dependent on Ca^{2+} influx. Notably, aloin failed to block the NMDA-induced inward currents in the absence of extracellular Ca^{2+} (NMDA: 26.9 ± 5.5 pA, aloin: 25.9 ± 6.2 pA, washout: 32.8 ± 7.9 pA, one-way ANOVA followed by Turkey's *post-hoc* test, $n = 10$, $p = 0.0001$, $F_{1.5, 13.2} = 23.2$; Fig. 5C). This finding indicates that although Na^+ and K^+ carry the majority of NMDAR-mediated current, extracellular Ca^{2+} modulates receptor activity and contributes to the total inward current. The observed reduction under Ca^{2+} -free conditions may also reflect enhanced Mg^{2+} blockade at -60 mV [17], but the disappearance of aloin's inhibitory effect suggests a Ca^{2+} -dependent mechanism of modulation.

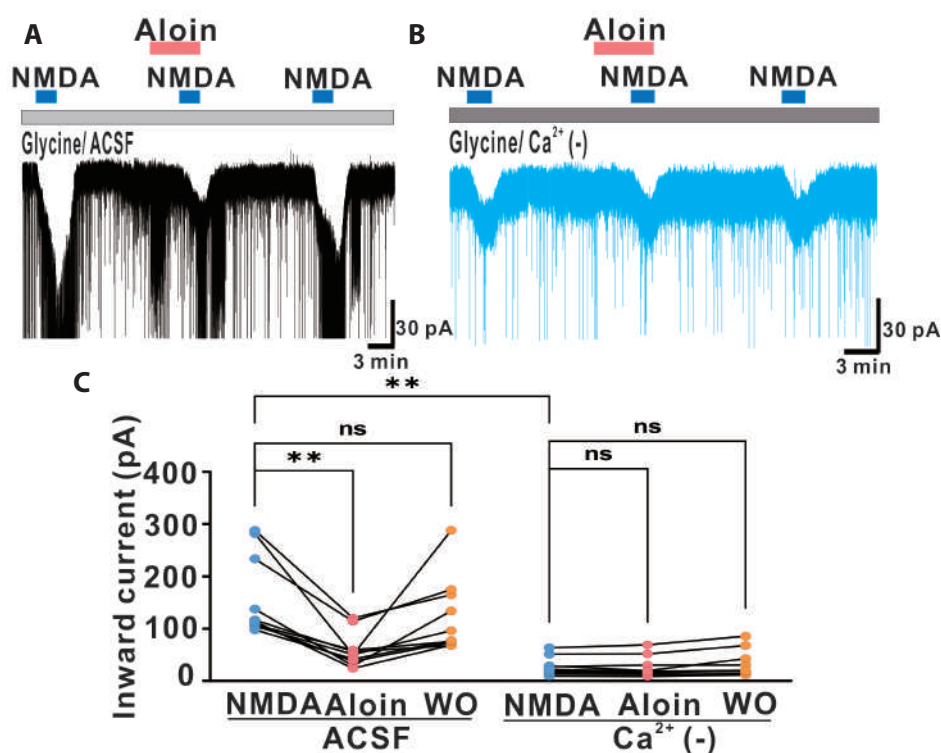


Fig. 5. Effects of aloin on *N*-methyl-D-aspartic acid (NMDA)-mediated responses in the absence of extracellular Ca^{2+} in substantia gelatinosa neurons. (A, B) Representative traces showing inhibition of NMDA-mediated responses by aloin (500 μM) in the presence and absence of extracellular Ca^{2+} , respectively. (C) A line series plot showing NMDA-induced inward currents suppressed by aloin in Ca^{2+} containing standard solution compared to extracellular Ca^{2+} lacking solution, $n = 10$, $**p < 0.01$, one-way ANOVA. ns, not significant; ACSF, artificial cerebrospinal fluid.

We further examined the inhibitory effect of aloin on NMDARs in a Na^+ -free ACSF solution to determine whether Na^+ influx contributes to its mechanism of action. To eliminate Na^+ involvement, NaCl was equimolarly replaced with choline chloride in the extracellular medium. Under these conditions, NMDA perfusion continued to elicit inward currents with a mean amplitude of 94.9 ± 25.3 pA (Supplementary Fig. 1). Notably, aloin-mediated suppression of NMDAR activity persisted in the Na^+ -free solution (aloin: 41.5 ± 9.6 pA; washout: 68.2 ± 14.8 pA; one-way ANOVA followed by Tukey's *post-hoc* test, $n = 8$, $p = 0.047$, $F_{1,2,8,5} = 5.1$; Supplementary Fig. 1B). These results indicate that aloin selectively inhibits Ca^{2+} influx through NMDARs in SG neurons of the Vc, independent of Na^+ influx.

Aloin's effect on calcium ion signaling

Fluorescence Ca^{2+} imaging was performed on transverse brainstem slices to confirm aloin's inhibitory effect on calcium signaling in the Vc region. Fig. 6B showed the time-intensity histogram of relative fluorescence intensity during experiment. Application of aloin (500 μM) reduced the NMDA-induced increase in fluorescence intensity, which partially recovered after washout. In fluorescence images of the laminae I-II region, fluorescence signal was visually reduced in the presence of aloin compared to before its application (Fig. 6C). More specifically, mean fluorescence intensity per pixel (Fig. 6D) was reduced from 82.6 arbitrary units (a.u.) to 76.2 a.u. in the absence and presence of aloin, respectively. The result of Ca^{2+} imaging experiment indicated that aloin could partially inhibit the Ca^{2+} influx in the Vc region.

DISCUSSION

Our study is the first to show that aloin suppresses hyperactivity of iGluRs in SG neurons of the Vc through patch-clamp electrophysiology. This inhibitory effect persisted even in the presence of TTX, suggesting that aloin acts directly on postsynaptic iGluRs. Specifically, aloin reduced NMDAR-mediated responses by modulating Ca^{2+} influx while leaving Na^+ conductance unchanged. Additionally, in Ca^{2+} imaging experiments of brain slice containing the Vc region, aloin reduced the increase of fluorescence signal intensity induced by NMDA. Furthermore, aloin significantly decreased the spontaneous firing of SG neurons, demonstrating its strong ability to regulate excitatory transmission. These findings suggested that the suppression of Ca^{2+} influx might underlie aloin's attenuation of NMDAR responses, potentially protecting SG neurons from excessive excitability.

Glutamate, the main excitatory neurotransmitter in the CNS, is vital for cognitive functions but can cause excitotoxicity via overactivation of iGluRs, including NMDARs, AMPARs, and KARs [3]. NMDAR overstimulation disrupts Ca^{2+} homeostasis, leading to neuronal death. In our study, aloin suppressed glutamate-induced responses in SG neurons [4]. This effect persisted in the presence of TTX, indicating a direct post-synaptic action on iGluRs. Although aloin is not a confirmed NMDAR inhibitor, related compounds like aloë-emodin inhibit NMDA-induced apoptosis by regulating ERK signaling [18], suggesting similar pathways may underlie aloin's effects. Beyond receptor modulation, aloin exhibits broader neuroprotective properties. It protects PC12 cells from oxygen-glucose deprivation by improving vi-

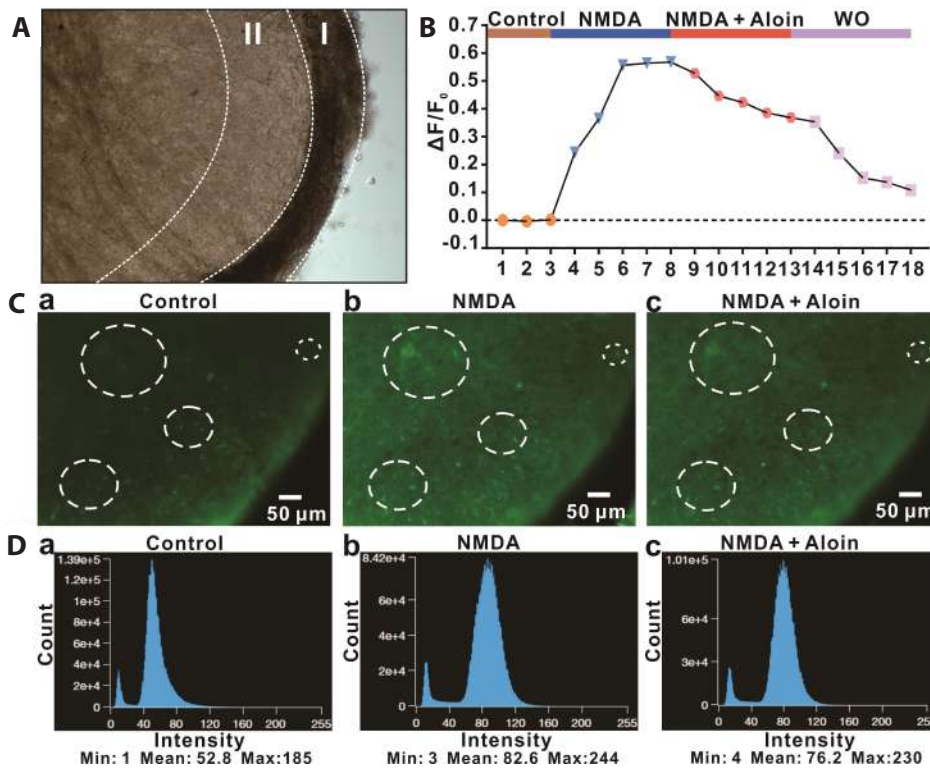


Fig. 6. Effects of aloin on Ca^{2+} signals on the Vc region. (A) Microscopic image of a brain slice highlighting the lamina I-II region. (B) Time-intensity histogram illustrating the reduction in fluorescence intensity during co-application of NMDA and aloin compared to NMDA alone, with recovery observed after washout. (C) Representative fluorescence images of brain slices stained with Fluo-3 AM, showing Ca^{2+} transients under different conditions: control (a), NMDA application (b), NMDA + aloin co-application (c). (D) Histogram depicting intensity distribution and mean pixel intensity for the three corresponding frames in panel C. Vc, trigeminal subnucleus caudalis; NMDA, N-methyl-D-aspartic acid.

ability, reducing oxidative stress, and regulating apoptosis-related genes [19,20]. Additionally, aloe arborescens extract and its fermented form mitigate glutamate-induced cytotoxicity in HT22 neurons by reducing reactive oxygen species (ROS), intracellular Ca^{2+} , and mitochondrial damage. The fermented extract showed enhanced effects due to increased levels of active compounds like aloin A, B, and aloenin [11]. These findings support aloin's potential to attenuate glutamate-induced neurotoxicity and highlight its promise as a natural therapeutic agent.

Calcium (Ca^{2+}) signaling is crucial for neuronal function, but its dysregulation underlies glutamate-induced excitotoxicity [21,22]. Excessive activation of NMDARs causes substantial Ca^{2+} influx, triggering harmful pathways such as mitochondrial permeability transition pore (mPTP) opening, leading to membrane depolarization, ATP depletion, and apoptosis [23,24]. Elevated Ca^{2+} also activates calpain, a protease that degrades cytoskeletal proteins, exacerbating neuronal damage [25]. In our study, aloin significantly suppressed NMDA-induced responses in SG neurons without altering NMDAR desensitization. This suppression was abolished in Ca^{2+} -free but not Na^{+} -free conditions, indicating a Ca^{2+} -specific mechanism. While most NMDAR-mediated current is conducted by Na^{+} and K^{+} , the functional modulation of NMDARs is strongly influenced by Ca^{2+} entry. Consistent with a study by Zhang *et al.* [17], our findings under Ca^{2+} -free conditions may partly result from increased Mg^{2+} block at hyperpolarized potentials. Nevertheless, the selective loss of aloin's inhibitory effect supports a Ca^{2+} -dependent regulatory component in NMDAR modulation. Similar to other aloe compounds, aloin may

regulate Ca^{2+} influx through NMDARs, contributing to neuroprotection. For example, aloe-emodin nanoparticles reduced ischemic damage by inhibiting astrocytic Ca^{2+} waves [5], while fermented aloe extract protected hippocampal neurons by reducing ROS and Ca^{2+} levels [11]. Calcium imaging using Fluo-3 AM confirmed that aloin attenuated NMDA-induced Ca^{2+} influx, consistent with electrophysiological data. These findings highlight aloin's role in modulating Ca^{2+} homeostasis and support its therapeutic potential against glutamate-induced neuronal injury.

In our study, aloin selectively inhibited NMDAR-mediated responses without affecting AMPAR- or KAR-mediated activity, despite their shared structural and pharmacological features as iGluRs [26,27]. This selectivity likely reflects the distinct structural and functional properties of NMDARs, which uniquely require co-activation by both glutamate and glycine [28,29]. To ensure full NMDAR activation, we co-applied glycine with NMDA, following established protocols [30]. NMDARs are highly permeable to Ca^{2+} , and their overactivation leads to excessive Ca^{2+} influx, a major driver of excitotoxic neuronal injury [31]. Aloin's inhibition of NMDARs may thus involve suppression of this Ca^{2+} entry. In contrast, AMPARs and KARs differ in subunit composition and gating, which may limit aloin's effects on them. This receptor-specific action underscores aloin's potential to selectively modulate NMDAR-mediated Ca^{2+} signaling and mitigate excitotoxic damage.

Although our study focused on a single effective concentration of aloin, future dose-response and time-course experiments will be important to establish the concentration threshold, maximal

efficacy, and temporal characteristics of aloin's effects on calcium signaling. In addition, mechanistic studies using selective blockers of voltage-gated calcium channels will help determine whether aloin's inhibitory effects are restricted to NMDAR-mediated Ca^{2+} influx or involve broader modulation of calcium channel activity. These investigations will further clarify the molecular basis of aloin's neuroprotective and anti-excitatory actions.

In conclusion, our study reveals that aloin selectively inhibits NMDAR-mediated responses in SG neurons while having no effect on AMPAR- and KAR-induced activities. This specificity suggests that aloin primarily targets NMDAR-dependent excitotoxic pathways, likely by modulating Ca^{2+} influx and downstream signaling mechanisms. Additionally, aloin significantly suppresses the spontaneous firing activity of SG neurons. Further research is needed to elucidate its precise molecular mechanisms and explore its therapeutic potential in neuroprotection.

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None to declare.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including one table can be found with this article online at <https://doi.org/10.4196/kjpp.25.317>

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