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ORIGINAL RESEARCH

Wrist-Ankle Acupuncture Alleviates Paclitaxel-Induced Neuropathic Pain in Mice by Neuronal α7 Nicotinic Acetylcholine Receptor–Dependent Modulation of Spinal Glutamatergic/NMDAR Signaling

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Background: Chemotherapy-induced peripheral neuropathy (CIPN) remains a major clinical challenge because effective mechanism-based therapies are limited. Wrist-ankle acupuncture (WAA) has shown analgesic benefits in CIPN, but the spinal mechanisms underlying its effects remain largely unclear.

Methods: Mechanical allodynia and thermal hyperalgesia were evaluated in a paclitaxel-induced CIPN mouse model. The cellular distribution of the α7 nicotinic acetylcholine receptor (α7nAChR) in the spinal dorsal horn was examined by immunofluorescence co-staining with NeuN-positive neurons. Spinal dorsal horn glutamate content and glutamatergic/N-methyl-D-aspartate receptor (NMDAR)-related signaling readouts (NMDAR NR2B subunit [NR2B], vesicular glutamate transporter 2 [VGluT2], phosphorylated c-Jun N-terminal kinase [p-JNK], phosphorylated cAMP response element-binding protein [p-CREB], and calcitonin gene-related peptide [CGRP]) were quantified using biochemical assays and Western blotting. To interrogate mechanism, an NMDAR agonist was administered intrathecally in a subset of mice prior to WAA. In parallel, neuronal α7nAChR was conditionally ablated via Cre–LoxP gene editing by delivering rAAV-hSyn-Cre into the lumbar dorsal horn of *Chrna7*$^{flox/flox}$ mice and by generating VGluT2 neuron–specific *Chrna7* conditional knockout mice (*Chrna7*$^{flox/flox}$; *Slc17a6*-Cre^{+}).

Results: WAA significantly attenuated mechanical allodynia and thermal hyperalgesia in CIPN mice. These behavioral improvements were accompanied by reduced dorsal horn glutamate levels and decreased NR2B/VGluT2-related measures and downstream markers (p-JNK, p-CREB, and CGRP), consistent with dampened glutamatergic/NMDAR pathway activity. Notably, intrathecal NMDAR activation or conditional loss of α7nAChR markedly blunted both the analgesic effect of WAA and its associated spinal molecular changes.

Conclusion: WAA alleviates paclitaxel-induced neuropathic pain in an α7nAChR-dependent manner, at least in part through modulation of spinal glutamatergic/NMDAR-related signaling.

Keywords: wrist-ankle acupuncture, chemotherapy-induced neuropathic pain, α7nAChR, spinal dorsal horn, glutamatergic/NMDAR-related signaling

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Introduction

Chemotherapy-induced neuropathic pain (CIPN) is a common and clinically significant adverse effect of cancer treatment, resulting from the neurotoxic properties of several chemotherapeutic agents and representing a major complication in modern oncology care.[1] Patients typically experience distal sensory disturbances, such as numbness and tingling in the distal extremities, along with mechanical allodynia and hyperalgesia.[2] These symptoms may persist for months to years after chemotherapy ends, and in some cases become a long-term burden that compromises daily function and quality of life.[3] Although substantial basic and clinical efforts have been devoted to understanding CIPN, effective mechanism-based therapies remain limited. A clearer understanding of the spinal and peripheral mechanisms driving CIPN is therefore needed to develop symptom-relieving strategies that do not compromise anticancer efficacy.

Wrist-ankle acupuncture (WAA), developed by Shuxin Zhang, is a modern acupuncture technique that applies stimulation at wrist and ankle regions based on traditional meridian concepts.[4,5] In contemporary practice and research settings, WAA is often delivered with low-frequency electrical stimulation to enhance analgesic efficacy. Emerging evidence suggests that WAA can reduce chronic pain symptoms, including neuropathic pain phenotypes relevant to CIPN.[6] For example, in myofascial pain syndrome, WAA has been reported to decrease pain intensity and alter cortical hemodynamic signals measured by near-infrared spectroscopy, implying that its analgesic actions may involve neural modulation rather than purely peripheral effects.[7] However, the spinal mechanisms that support WAA-mediated analgesia in CIPN remain insufficiently defined.

A key feature of CIPN is central sensitization within the spinal dorsal horn, where glutamatergic transmission and N-methyl-D-aspartate receptor (NMDAR)-related signaling contribute to persistent nociceptive processing. Accordingly, reducing dorsal horn glutamate availability and dampening glutamatergic/NMDAR pathway activity are considered important therapeutic directions.[8] Cholinergic signaling through nicotinic acetylcholine receptors can modulate dorsal horn synaptic transmission and pain processing, suggesting a potential interface between cholinergic receptors and glutamatergic signaling in CIPN.[9] Among these receptors, the α7 nicotinic acetylcholine receptor (α7nAChR) is widely expressed in the central nervous system and has been implicated as a promising target in chemotherapy-related neuropathology. In paclitaxel- and oxaliplatin-induced models, α7nAChR agonists have been reported to alleviate pain behaviors and reduce nerve fiber loss.[10,11]

Based on these observations, we hypothesized that WAA alleviates paclitaxel-induced CIPN by engaging spinal neuronal α7nAChR to modulate dorsal horn glutamatergic/NMDAR-related signaling. To test this hypothesis, we combined behavioral pain assays with genetic/viral manipulation of α7nAChR and pharmacological interrogation of NMDAR signaling to define the contribution of this pathway to WAA-induced analgesia.

Materials and Methods

Animals

This study used three mouse lines: *Chrna7*$^{flox/flox}$ (α7nAChR floxed) mice, *Slc17a6*(VGluT2)-Cre driver mice, and C57BL/6 wild-type (WT) mice. *Chrna7*$^{flox/flox}$ mice were purchased from The Jackson Laboratory (B6(Cg)-*Chrna7*$^{tm1.1Ehs}$/YakelJ; Stock No. 026965), and *Slc17a6*(VGluT2)-Cre mice were obtained from Guangzhou Yodoo Biotechnology Co., Ltd. (Stock No. GAP1008). Mice were housed and bred under specific pathogen-free (SPF) conditions at the Experimental Animal Center of Fujian University of Traditional Chinese Medicine, with ad libitum access to food and water, at 22–26°C, 40–50% humidity, and a 12-h light/dark cycle. For experiments, the following cohorts were used: (1) *Chrna7*$^{flox/flox}$ mice and their littermate flox-negative controls (*Chrna7*$^{+/+}$); (2) SPF-grade C57BL/6 WT mice; and (3) conditional knockout mice [*Chrna7*$^{flox/flox}$*;Slc17a6*-Cre^{+}, referred to as α7-cKO(VGluT2)] generated by crossing *Chrna7*$^{flox/flox}$ and *Slc17a6*-Cre mice, together with their littermate Cre-negative controls (*Chrna7*$^{flox/flox}$*; Slc17a6*-Cre^{-}).

Except for the saline group, paclitaxel (PTX)-induced CIPN was established as previously described by Toma et al[12] Briefly, a 5 mg/mL PTX (MedChemExpress) stock solution was prepared in 10% DMSO and 90% (20% sulfobutylether-β-cyclodextrin [SBE-β-CD] in saline; MedChemExpress). Immediately before each injection, the stock was diluted with sterile 0.9% sodium chloride (NaCl) to a working concentration of 0.2 mg/mL. Mice received intraperitoneal (i.p.) injections of PTX (2 mg/kg) every other day for a total of four injections. Vehicle controls received the same solvent mixture, diluted with sterile 0.9% NaCl to a final concentration of 33.3%.

All experimental procedures were approved by the Animal Ethics Committee of Fujian University of Traditional Chinese Medicine (Animal Ethics Approval No. FJTCM-IACUC-2021151) and were conducted in strict accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to reduce their suffering.

Intrathecal Injection

After successful modeling in the NMDA+WAA group, intrathecal injections of the NMDA agonist (1 μg/μL, 10 μL, i.t) were administered at a dose of 30 minutes prior to WAA sessions on days 7, 10, and 13.[13] The NMDA agonist used was N-Methyl-D-aspartic acid (NMDA, chemical name: N-Methyl-D-aspartic acid, CAS No.: 6384–92-5, Cat. No.: HY-17551, purity: 99.73%; MedChemExpress). The intrathecal injection method, adapted from previous studies,[14] involved the following steps: Adult male C57BL/6 mice (8–12 weeks, weighing 20–25 g) were anesthetized with isoflurane (R510-22-10, RWD Life Science, China) inhalation (oxygen flow rate: 0.5–1 L/min; induction concentration: 3%-5%, maintenance concentration: 1–2%) to maintain a stable respiratory rate (50–70 breaths per minute). The dorsal fur of the mice was shaved, and they were placed in a prone position on the operating table with the abdomen elevated to fully expose the L4-L5 intervertebral space. After disinfecting the area, a 27 G microsyringe (Hamilton) was vertically inserted into the spinal column and tilted at a 45° angle into the intervertebral space. Once resistance was lost, indicating proper placement, NMDA agonist was injected intrathecally, followed by a 30s pause before slowly withdrawing the syringe.

Intraspinal Microinjection

Intraspinal microinjection of rAAV-Cre virus into the lumbar spinal dorsal horn was performed to achieve region-specific, neuron-targeted deletion of *Chrna7* within the spinal pain-processing circuitry, rather than in dorsal root ganglia (DRG), because our study focused on the central (spinal) component of α7nAChR-mediated analgesia. The lumbar enlargement (L3–L4 spinal segments, corresponding to the sciatic nerve territory relevant to hindlimb behavioral testing) was selected as the target region. The procedure was performed with minor modifications based on the method described by Peterson et al[15] Mice were anesthetized with isoflurane (R510-22-10, RWD Life Science, China; induction 3–5%, maintenance 1–2% in oxygen at 0.5–1.0 L/min) and placed on a stereotaxic frame (RWD 68000, RWD Life Science, China). After confirming an adequate depth of anesthesia, a midline incision was made and a limited laminectomy was performed at the vertebral level overlying the lumbar enlargement (L3-L4 spinal segments). The dura was carefully kept intact and the spinal cord was stabilized to minimize respiratory motion. A pulled glass micropipette was connected to a 10 μL Hamilton microsyringe and loaded with rAAV-hSyn-SV40-NLS-Cre (titer $\geq 2.05\times10^{12}$ vg/mL).

Adult male *Chrna7*$^{flox/flox}$ mice were used for intraspinal delivery of rAAV-Cre to induce region-specific deletion of *Chrna7* in the spinal dorsal horn, as α7-cKO (AAV-Cre). The injector was mounted on a microinjection pump (RWD, China), and virus was delivered at 50 nL/min into the dorsal horn at the following coordinates relative to the spinal cord midline: 0.25 mm lateral to the midline and 0.30 mm ventral from the dorsal surface. A total volume of 500 nL was injected per side (bilateral injections), and the micropipette was left in place for 10 min after each injection to allow diffusion and to minimize reflux before slow withdrawal. Post-surgery, the incision was sutured, antibiotics were applied, and analgesics were administered. The mice were kept warm and observed for 2–3 days before being returned to standard housing conditions for 21 days to prepare for subsequent experiments.

Wrist-Ankle Acupuncture Treatment

Starting from day 7 after paclitaxel-induced CIPN model establishment, adult male mice received weak electrical stimulation acupuncture at bilateral "lower 4" and "lower 5" points for 30 minutes daily for 7 consecutive days. The specific anatomical locations were defined as follows: Lower 4 point: Midpoint between the anterior border of the tibia and anterior border of the fibula, 1–2 mm proximal to the tip of the lateral malleolus. Lower 5 point: Midline of the lateral calf, along the posterior border of the fibula, 1–2 mm proximal to the tip of the lateral malleolus. Acupuncture needles (0.5 inch) were inserted at a 15-degree angle along the skin surface to a depth of 2–3 mm. The mice were maintained under light anesthesia with 0.5–1.0% isoflurane inhalation and fixed on a heating pad to maintain body temperature throughout the procedure. Needle tips were connected to an acupoint nerve stimulator (HAN'S-200A, China) with the following parameters: frequency set at 2/100 Hz (alternating dense-

sparse waves), and intensity at 1 mA. The 2/100 Hz alternating dense-sparse wave frequency was selected based on established protocols for electroacupuncture analgesia, as this waveform has been shown to activate multiple opioid receptor subtypes and provide broader analgesic coverage compared with single-frequency stimulation.[16,17] The 1 mA intensity was chosen as a standard, sub-nociceptive level consistent with prior WAA and electroacupuncture studies in mice.[18]

Behavioral Tests

Von Frey Test

The paw withdrawal threshold (PWT) for mechanical allodynia was evaluated in mice using the "up-and-down" method described by Chaplan S R.[19] The Von Frey filaments of varying intensities were applied to the plantar surface of the hind paw, and withdrawal responses were recorded. Mice were acclimated to the testing environment for 30 minutes before assessments. Positive responses, such as foot lifting, shifting, or licking upon filament application, were noted. Starting with the first negative response followed by a positive response, at least four additional force values were tested. The 50% Von Frey response threshold formula was used to calculate the final PWT, reflecting the mechanical pain behavior of the mice:

$$\mathrm{PWT\ (g)} = 10^{(\mathrm{Xf} + \mathrm{k}\delta)} / 10000$$

In this formula, Xf is the $\log_{10}$ value of the final filament used, k is a tabular value based on the response pattern, and δ is the mean difference between stimulus intensities (0.224 for the standard set).

Hargreaves Test

Following the study by Yeomans DC,[20] paw withdrawal latency (PWL) for hyperalgesia was assessed in mice using a radiant heat apparatus. The procedure was as follows: Mice were placed in transparent plastic cages and allowed to acclimate for 30 min. Once the mice were calm, the central area of the hind paw's skin was stimulated using the apparatus' radiant heat source, avoiding the toe pad area. When the mice withdrew, licked, or shook their paw in response to the heat, the infrared stimulus light automatically shut off, displaying the PWL. The parameters were kept consistent throughout the experiment: stimulus intensity was fixed at 30%, the maximum duration of heat radiation was limited to 30s to prevent tissue damage, and the cage bottoms were kept dry to avoid interference from humidity. Each site was measured five times at 5 min intervals, and the average of the five PWL measurements was calculated as the thermal pain latency for each mouse.

Measurement of Glutamate Content

The glutamate content was measured in L4–L6 spinal dorsal horn homogenates using a glutamate assay kit (Solarbio, China). Tissue samples were obtained after the animals were deeply anesthetized with an intraperitoneal injection of Avertin (ready-to-use tribromoethanol; MI00681, Mishu, China) at 20 μL/g body weight and euthanized by cervical dislocation. Tissue samples were homogenized in Reagent 1 (provided in the kit) at a ratio of 1 g tissue to 5 mL reagent under ice-bath conditions. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was collected and kept on ice for immediate analysis. Glutamate concentration was determined by comparing absorbance values measured at 340 nm with the calibration curve of standard solutions, and all subsequent procedures were performed strictly according to the manufacturer's instructions (Solarbio, China).

Western Blotting

After deep anesthesia was induced in mice by intraperitoneal injection of Avertin (ready-to-use tribromoethanol; MI00681, MMouse, China) at 20 μL/g body weight, the animals were euthanized by cervical dislocation according to the approved animal protocol. L4–L6 spinal dorsal horn samples were then rapidly collected and lysed at low temperature in a buffer containing a protease/phosphatase inhibitor cocktail (MedChemExpress, China). Proteins were separated on a 10% PAGE gel (Epizyme Biotech, China) and transferred onto a PVDF membrane (Millipore, Billerica, MA). After 1 hour of incubation with rapid blocking solution (Beyotime Biotech, China) at room temperature, the membrane was washed with TBST and incubated with primary antibodies overnight at 4°C. The primary antibodies used were: Rabbit anti-α7nAChR (Abcam, 1:1000), Rabbit anti-NR2B (Proteintech, 1:1000), Mouse anti-VGluT2 (Abcam, 1:1000), Rabbit anti-p-JNK (Abcam, 1:500), Rabbit anti-JNK (CST, 1:2000), Rabbit anti-p-CREB (Proteintech, 1:1000), Rabbit anti-CREB (Proteintech, 1:2000), Rabbit anti-CGRP

(Proteintech, 1:1000), Rabbit anti-GAPDH (Proteintech, 1:8000). After washing, the membrane was incubated with HRP-conjugated secondary antibodies (Beyotime Biotech; goat anti-rabbit, 1:10000; goat anti-mouse, 1:10000) at room temperature for 1 hour. Protein bands were visualized using an Enhanced Chemiluminescence detection system (Bioscience, China), with GAPDH as the loading control. Phosphorylation indexes were normalized to total protein levels. Western blot images were captured using the ChemiDoc XRS$^+$ chemiluminescence imaging system (BioRad), and gray values were measured using ImageJ software.

Immunofluorescence

Mice were deeply anesthetized by intraperitoneal injection of Avertin (ready-to-use tribromoethanol; MI00681, MMouse, China) at 20 μL/g body weight. Animals were transcardially perfused with saline followed by 4% paraformaldehyde (PFA). The L4-L6 spinal cord segments were collected, post-fixed in 4% PFA, sunk in 15% and 30% sucrose solutions, embedded in OCT (Sakura, Japan), and ectioned at 10 μm. Immunofluorescence staining was performed using a tyramide signal amplification (TSA) kit (Aifang Biological,China). Sections were thawed at room temperature, incubated in 3% hydrogen peroxide for 15 minutes, and washed with PBST. After blocking with normal goat serum for 30 minutes, sections were incubated overnight at 4°C with rabbit anti-α7nAChR antibody (Abcam, 1:2000). The next day, sections were washed and incubated with poly HRP-conjugated goat anti-rabbit/mouse secondary antibody for 50 minutes, and then treated with 570-TSA fluorescent dye for 5 minutes. After antibody elution at 37°C for 5 min, followed by thorough washes and re-blocking. Sections were then incubated overnight at 4°C with either mouse anti-NeuN (Proteintech, 1:2000) or rabbit anti-VGluT2 (Abcam, 1:1000). After washing, sections were incubated with Poly-HRP secondary antibody for 50 min and developed with TSA-488 fluorophore for 5 min. Finally, sections were mounted with anti-fade medium containing DAPI and imaged using a fluorescence microscope (Leica DMI8).

Statistical Analysis

Data statistical analysis was conducted using SPSS software (version 23.0). Data are presented as mean ± SEM. Normality was assessed prior to parametric testing. Independent-samples t-tests were used for two-group comparisons (eg., α7nAChR deletion vs Control). For PWT and PWL data, repeated measures analysis of variance (ANOVA) was conducted. If the assumption of sphericity was met ($P > 0.05$), the significance of the sphericity assumption was primarily considered in the within-subjects effects test. Otherwise, results corrected by Greenhouse-Geisser were used. Single-factor analysis of variance was applied to compare multiple groups, followed by post-hoc tests for pairwise comparisons. If homogeneity of variances was met, the LSD method was used for pairwise comparisons. If variances were not homogeneous, the Games-Howell method was employed. Differences were considered statistically significant at $P < 0.05$.

Results

Neuronal α7nAChR Is Required for WAA Alleviated Paclitaxel-Induced CIPN

Previous studies have reported that α7nAChR is predominantly expressed in neurons within spinal dorsal horn.[21] Consistent with this, our co-localization analysis showed that α7nAChR immunoreactivity largely overlapped with NeuN$^+$ neurons, with minimal overlap with Iba1$^+$ microglia or GFAP$^+$ astrocytes (see Supplementary Figure 1). The overall experimental design is summarized in Figure 1a. Following four intraperitoneal paclitaxel injections, α7nAChR protein levels in the spinal dorsal horn decreased compared with saline group (Figure 1g–j). To examine the functional contribution of neuronal α7nAChR, we selectively deleted *Chrna7* in dorsal horn neurons using a Cre–LoxP approach. Specifically, rAAV-hSyn-SV40-NLS-Cre was microinjected bilaterally into the lumbar dorsal horn of *Chrna7*$^{flox/flox}$ mice. At 21 days post-injection, spinal dorsal horns tissue from the Control and α7-cKO (AAV-Cre) groups were collected to confirm deletion efficiency. Western blotting and immunofluorescence confirmed a marked reduction in α7nAChR expression in the α7-cKO (AAV-Cre) group relative to Control group (Figure 1b–e).

Behaviorally, paclitaxel-treated mice developed both mechanical allodynia and thermal hyperalgesia, as evidenced by a significant decrease in the paw withdrawal threshold (PWT, g) to von Frey filament stimulation and a marked reduction in the paw withdrawal latency (PWL, s) to a radiant heat source. These pain hypersensitivities were accompanied by

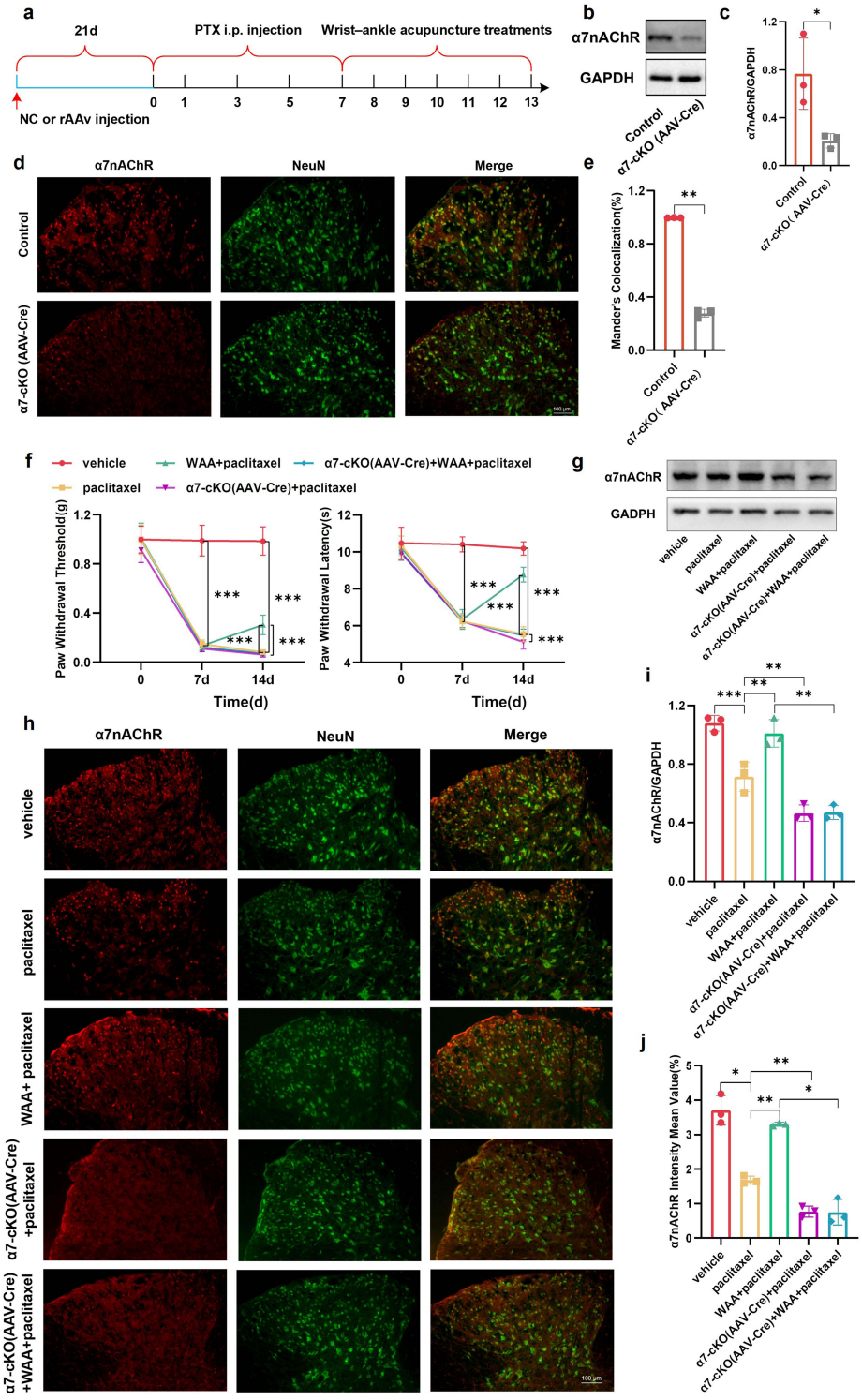

Figure 1 WAA alleviates paclitaxel-induced mechanical allodynia and thermal hyperalgesia in a neuronal α7nAChR–dependent manner. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (**a**) Experimental design and timeline for the *Chrna7*$^{flox/flox}$ mouse cohort; detailed procedures are provided in the Methods. (**b**) Representative Western blot bands of α7nAChR in the spinal dorsal horn. (**c**) Quantification of α7nAChR protein levels 21 days after lumbar dorsal horn microinjection of control virus or rAAV-hSyn-SV40-NLS-Cre (N=3, t = 3.2, P = 0.033). (**d**) Representative immunofluorescence images showing colocalization of α7nAChR (red) and NeuN (green); scale bar: 100 μm. (**e**) Quantification of neuronal association/targeting using Manders' overlap coefficient (fraction of α7nAChR overlapping NeuN, N=3, t = 41.833, P = 0.001). (**f**) Behavioral assessments of mechanical (paw withdrawal threshold, PWT) and thermal sensitivity (paw withdrawal latency, PWL) at days 0, 7, and 14; WAA-induced analgesia was attenuated after dorsal horn neuronal α7nAChR knockdown (N=9,day 14: F_{PWT}=342.009, P_{PWT} < 0.001; F_{PWL}=333.245, P_{PWL} < 0.001). (**g**) Representative Western blot bands of α7nAChR in the spinal dorsal horn across indicated groups following paclitaxel administration and WAA treatment. (**h**) Densitometric quantification of α7nAChR protein levels corresponding to panel (**g**) (N=3, F=46.729, P < 0.001). (**i**) Representative immunofluorescence images of α7nAChR (red) and NeuN (green) in the spinal cord dorsal horn on day 14 after model induction; scale bar: 100 μm. (**j**) Quantification of α7nAChR immunofluorescence intensity (mean intensity), corresponding to panel (**i**) (N=3, F=81.123, P < 0.001).

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a decreased expression of α7nAChR in the spinal dorsal horn at the corresponding time point (Figure 1f, g–j). WAA treatment significantly alleviated paclitaxel-induced pain behaviors and restored α7nAChR levels in the dorsal horn. Importantly, neuronal *Chrna7* deletion induced by spinal rAAV-Cre largely abolished the analgesic benefit of WAA (Figure 1f). Together, these findings indicate that neuronal α7nAChR in the spinal dorsal horn is required for the analgesic effect of WAA in this paclitaxel-induced CIPN model.

Neuronal α7nAChR Mediates the Modulatory Effect of WAA on Spinal NMDAR Signaling Activity

This study aimed to evaluate whether WAA modulates spinal glutamatergic/NMDAR signaling activity in paclitaxel-induced CIPN. Quantification of glutamate levels in the spinal dorsal horn demonstrated a significant reduction following WAA treatment (Figure 2a). Concurrently, assessment of neuronal activation markers indicated that WAA reduced c-fos immunoreactivity in the dorsal horn (see Supplementary Figure 2). Western blotting further demonstrated that WAA lowered the expression of several molecular readouts associated with enhanced excitatory signaling in this region. Notably, these WAA-associated changes were largely diminished when α7nAChR was conditionally knocked down, supporting the interpretation that the effects of WAA on spinal glutamatergic/NMDAR pathway activity—and its behavioral analgesic benefit—are dependent, at least in part, on neuronal α7nAChR (Figure 2b–g).

Intrathecal NMDAR Activation Attenuates the Analgesic Effect of WAA in Paclitaxel-Induced CIPN

To confirm whether NMDAR signaling is required for the analgesic effect of WAA in paclitaxel-induced CIPN (Figure 3a), an NMDAR agonist was administered intrathecally 30 min before WAA treatment on days 7, 10, and 14 after the first paclitaxel injection. PWT and PWL assessments indicated that NMDAR activation substantially reduced the behavioral analgesic benefit of WAA (Figure 3b). Consistently, NMDAR agonist administration diminished WAA-associated changes in molecular readouts of spinal glutamatergic/NMDAR signaling activity in the dorsal horn, including NR2B- and VGluT2-related measures and downstream signaling markers (eg., p-CREB, p-JNK, and CGRP) (Figure 3c–i). These findings support a model in which WAA alleviates CIPN partly by modulating spinal glutamatergic/NMDAR signaling.

α7nAChR in VGluT2-Expressing Neurons Is Required for the Analgesic and Spinal Glutamatergic/NMDAR-Modulatory Effects of WAA in Paclitaxel-Induced CIPN

To determine whether α7nAChR in glutamatergic neurons is required for the analgesic effects of WAA (Figure 4a), we generated VGluT2 neuron–specific *Chrna7* conditional knockout mice using the Cre–LoxP system [*Chrna7*$^{flox/flox}$; Slc17a6-Cre^{+}, referred to as α7-cKO (VGluT2)] and compared them with littermate Cre-negative controls (*Chrna7*$^{flox/flox}$; Slc17a6-Cre^{-}, referred to as Control). Western blotting confirmed a significant reduction of α7nAChR protein in the spinal dorsal horn of α7-cKO (VGluT2) mice relative to controls (Figure 4b and c). Consistently, immunofluorescence showed that α7nAChR immunoreactivity in the dorsal horn overlapped with VGluT2^{+} neurons in Control mice and was markedly reduced in α7-cKO (VGluT2) mice (Figure 4d and e).

Behavioral assessments demonstrated that VGluT2 neuron–specific *Chrna7* deletion substantially attenuated the analgesic effects of WAA on paclitaxel-induced mechanical allodynia and thermal hyperalgesia (Figure 4f). In parallel, the ability of WAA to reduce dorsal horn glutamate levels was diminished in α7-cKO (VGluT2) mice (Figure 4g). Furthermore, WAA reduced several molecular associated with enhanced spinal glutamatergic/NMDAR pathway activity after paclitaxel administration, including NR2B- and VGluT2-related measures and downstream signaling markers (p-JNK, p-CREB, and CGRP). Notably, these WAA-associated changes were largely blunted in VGluT2 neuron–specific α7-cKO (VGluT2) mice (Figure 4h–m). These data indicate that α7nAChR expressed in VGluT2^{+} glutamatergic neurons contributes critically to WAA-induced modulation of spinal glutamatergic/NMDAR signaling activity and the associated behavioral analgesia in this paclitaxel-induced CIPN model.

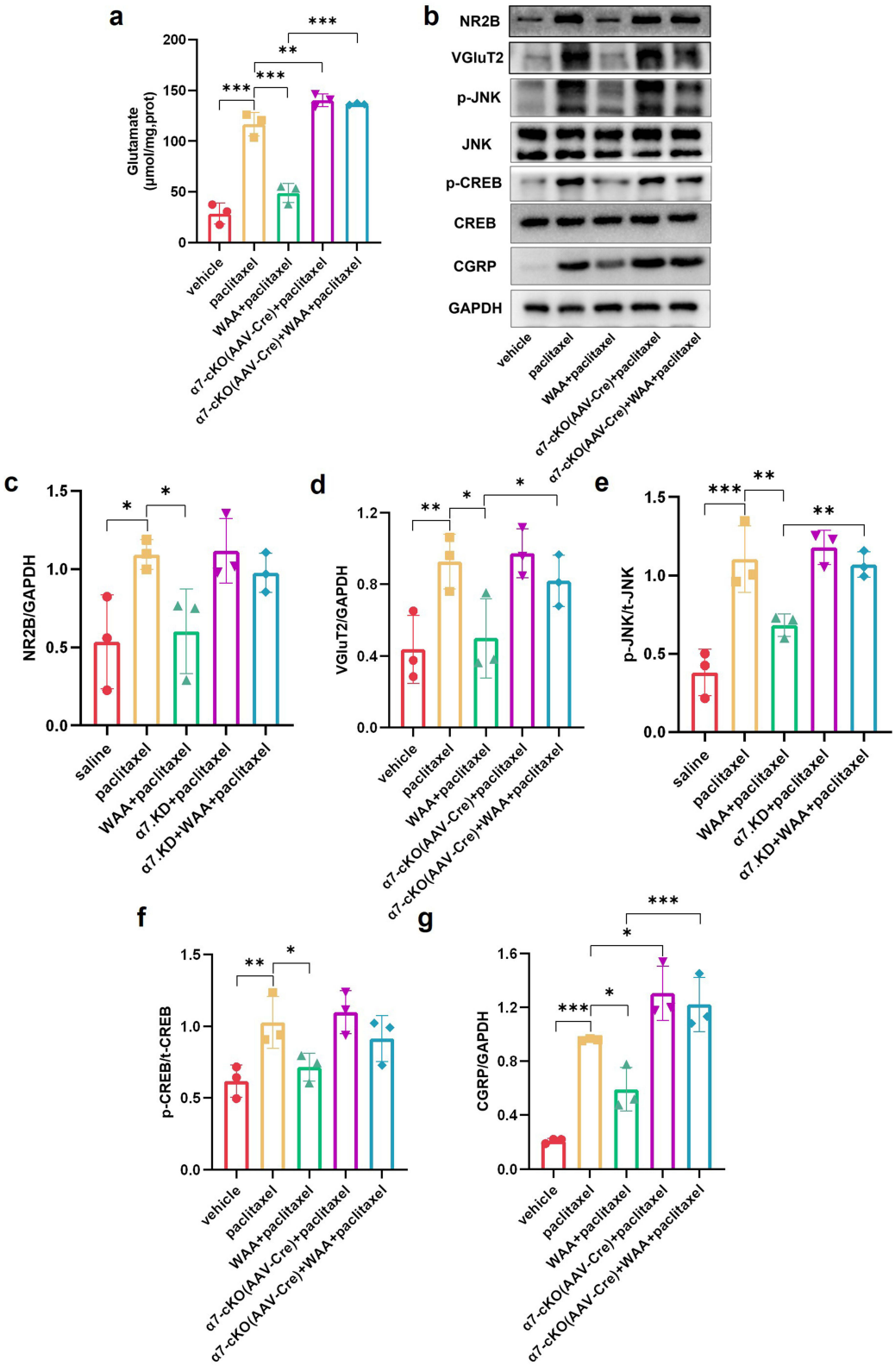

Figure 2 WAA reduces spinal glutamate levels and glutamatergic/NMDAR-related signaling readouts in an α7nAChR-dependent manner. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**a**) Glutamate (glutamic acid) content in the spinal dorsal horn. (N=3, F=109.563, P< 0.001). (**b**) Representative Western blot bands of proteins associated with glutamatergic/NMDAR pathway activity in the spinal dorsal horn. (**c**–**g**) Densitometric quantification of NR2B (**c**), VGluT2 (**d**), p-JNK(**e**) p-CREB (**f**) and CGRP(**g**) in the spinal cord dorsal horn. (N=3, NR2B: F=4.955, $P < 0.05$; VGluT2: F=6.219, $P < 0.01$; p-JNK: F=19.273, P<0.001; p-CREB: F=5.195, $P < 0.05$; CGRP: F=28.823, $P < 0.001$).

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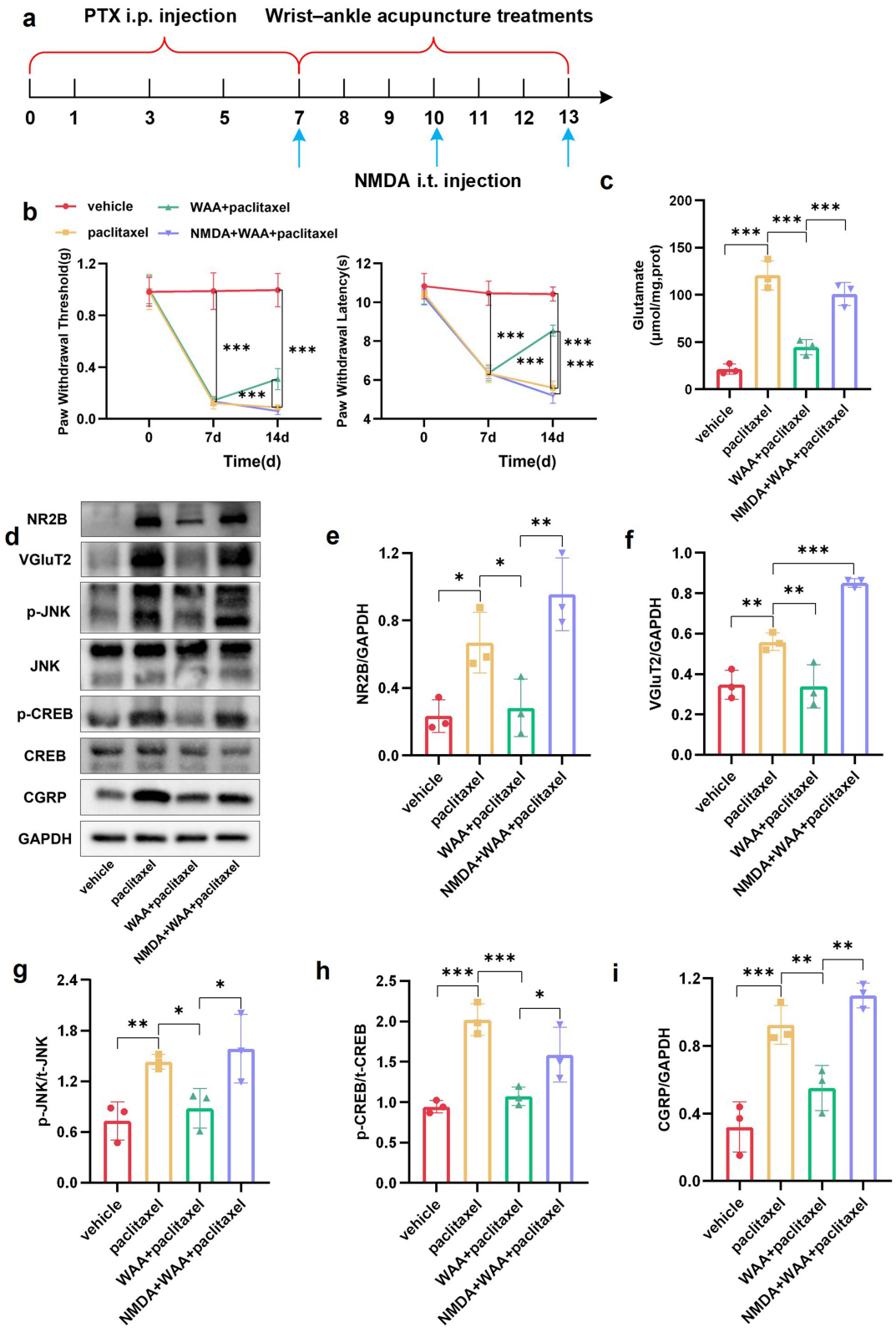

Figure 3 Intrathecal NMDAR activation attenuates the analgesic and spinal molecular effects of WAA in paclitaxel-induced CIPN. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**a**) Experimental design and timeline for the C57BL/6 cohort; detailed procedures are provided in the Methods. (**b**) Behavioral assessment of PWT and PWL at days 0, 7, and 14. Intrathecal administration of an NMDAR agonist prior to WAA reduced the behavioral analgesic benefit of WAA. (N=9; two-way repeated-measures ANOVA; day 14: F_{PWT}=281.188, $P_{PWT} < 0.001$; F_{PWL}=461.233, $P_{PWL} < 0.001$). (**c**) Glutamate (glutamic acid) content in the spinal dorsal horn. (N=3, F=54.286, $P < 0.001$). (**d**) Representative Western blot bands of proteins associated with glutamatergic/NMDAR pathway activity in the spinal dorsal horn. (**e–i**) Densitometric quantification of NR2B (**e**) VGluT2 (**f**) p-JNK (**g**) p-CREB (**h**) and CGRP (**i**) in the spinal cord dorsal horn.(N=3, NR2B: F=11.877, $P < 0.01$; VGluT2: F=36.553, $P < 0.001$; p-JNK: F=8.216, $P < 0.01$; p-CREB: F=16.911, $P < 0.01$; CGRP: F=25.487, $P < 0.001$).

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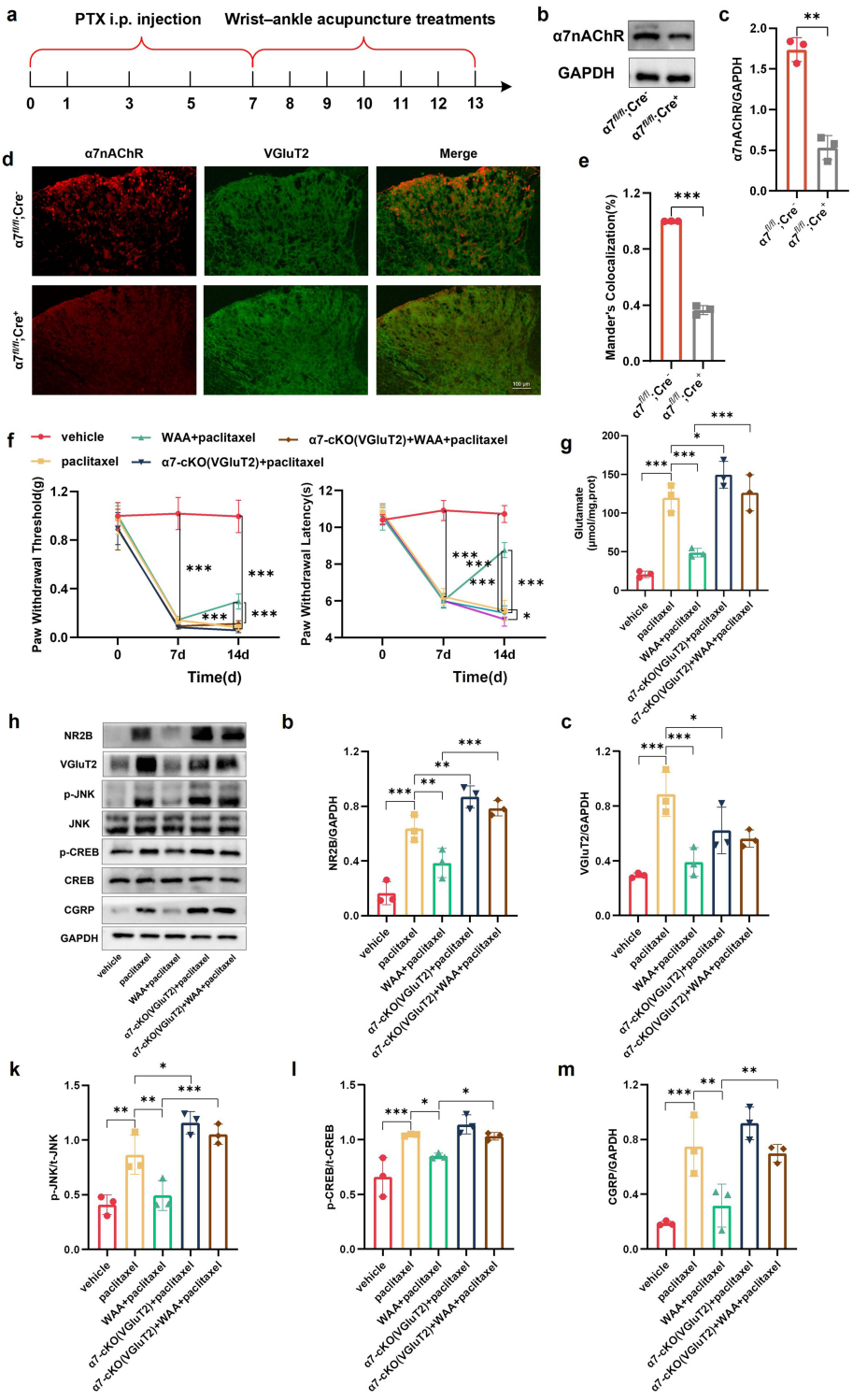

Figure 4 α7nAChR in VGluT2-expressing glutamatergic neurons is required for the analgesic and spinal glutamatergic/NMDAR-modulatory effects of WAA in paclitaxel-induced CIPN. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (**a**) Experimental design and timeline for VGluT2 neuron–specific *Chrna7* conditional knockout mice (*Chrna7*$^{flox/flox}$;*Slc17a6*-Cre^{+}, α7-cKO(VGluT2)) and littermate Cre-negative controls (*Chrna7*$^{flox/flox}$;*Slc17a6*-Cre^{-}); detailed procedures are provided in the Methods. (**b**) Representative Western blot bands of α7nAChR in the spinal dorsal horn. (**c**) Densitometric quantification of α7nAChR protein levels in α7-cKO(VGluT2) mice and controls (N=3, t=9.954, P=0.001). (**d**) Representative immunofluorescence images of the spinal dorsal horn showing α7nAChR (red) and VGluT2 (green); scale bar, 100 μm. (**e**) Quantification of α7nAChR immunofluorescence intensity (mean intensity) corresponding to panel (**d**). (N=3, t=33.319, P < 0.001). (**f**) Behavioral assessment of PWT and PWL at days 0, 7, and 14. VGluT2 neuron–specific α7nAChR deletion attenuated the analgesic effects of WAA (N=9; two-way repeated-measures ANOVA; day 14: F_{PWT}=308.427, P_{PWT} < 0.001; F_{PWL}=301.306, P_{PWL} < 0.001). (**g**) Glutamate (glutamic acid) content in the spinal dorsal horn. (N=3, F=37.871, P<0.001). (**h**)Representative Western blot bands of proteins associated with glutamatergic/NMDAR pathway activity in the spinal dorsal horn. (**i–m**) Densitometric quantification of NR2B (**i**) VGluT2 (**j**) p-JNK (**k**) p-CREB (**l**) and CGRP (**m**) in the spinal dorsal horn (N=3, NR2B: F=36.601, P < 0.001; VGluT2: F=11.265, P < 0.001; p-JNK: F=21.336, P < 0.001; p-CREB: F=13.144, P < 0.01; CGRP: F=15.896, P < 0.001).

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Discussion

Chemotherapy-induced peripheral neuropathy (CIPN) is a prevalent and dose-limiting complication of paclitaxel, severely impacting quality of life and treatment continuity.[22,23] As cancer survival improves, the long-term burden of CIPN is becoming increasingly prominent.[24,25] In this context, non-pharmacological strategies that can mitigate neuropathic pain without compromising antitumor efficacy are highly desirable. Despite progress in supportive care, mechanism-based options for CIPN remain limited. Here, we show that paclitaxel-induced hypersensitivity is accompanied by reduced α7nAChR expression in the spinal dorsal horn, and that WAA alleviates pain behaviors in an α7nAChR-dependent manner while reducing glutamate levels and NMDAR-related signaling readouts. A central observation of our work is that paclitaxel-induced CIPN was associated with reduced α7nAChR expression in the spinal dorsal horn, whereas WAA increased α7nAChR levels and improved mechanical and thermal pain hypersensitivity. This association was functionally supported by two complementary approaches: (i) viral-mediated, neuron-targeted conditional reduction of α7nAChR in the dorsal horn, which reversed the analgesic benefit of WAA; and (ii) cell type–specific genetic deletion of α7nAChR in VGluT2-expressing excitatory neurons, which similarly attenuated WAA-induced analgesia. Together, these findings strengthen the inference that neuronal α7nAChR is not merely a correlate, but a key contributor to the spinal mechanism by which WAA modulates CIPN-related hypersensitivity.

CIPN has been consistently linked to spinal dorsal horn sensitization, where enhanced glutamatergic drive and NMDAR signaling facilitate persistent nociceptive processing.[26] We observed that paclitaxel increased dorsal horn glutamate levels and elevated multiple molecular markers commonly used to index enhanced excitatory signaling, including NR2B, VGluT2, and downstream activity-associated markers such as p-JNK, p-CREB, and CGRP. These changes occurred alongside lowered behavioral thresholds, consistent with a sensitized spinal state. WAA reduced glutamate content and decreased the expression of these markers, and also diminished c-fos immunoreactivity, suggesting reduced dorsal horn neuronal activation. While these measures do not directly quantify neuronal firing or synaptic currents, the convergence of biochemical and activity-related readouts supports the interpretation that WAA dampens spinal glutamatergic/NMDAR pathway activity associated with CIPN hypersensitivity.

The involvement of NMDAR signaling in WAA-mediated analgesia was further supported by pharmacological probing. Intrathecal administration of an NMDAR agonist prior to WAA markedly reduced WAA's behavioral analgesic effect and weakened its associated changes in dorsal horn molecular readouts. These results suggest that WAA engages spinal NMDAR/NR2B-related nociceptive signaling and plasticity. The direction of these effects is consistent with therapeutic strategies that dampen NR2B-dependent signaling, although WAA is not equivalent to pharmacological NR2B antagonism.[27] This aligns with prior work showing that blocking NMDARs can reverse chemotherapy-induced hypersensitivity and normalize excitatory synaptic transmission in dorsal horn neurons.[28] Conversely, augmenting NMDAR signaling can be expected to bias the system toward sensitization, thereby counteracting analgesic interventions. In this context, our findings place NMDAR-related signaling as a relevant mechanistic node for WAA's action in paclitaxel-induced CIPN.

Among NMDAR components, NR2B is a well-recognized determinant of channel properties and pain-related plasticity.[29] Prior studies have often emphasized increased phosphorylation of synaptic GluN2B subunits in neuropathic pain states.[30,31] Notably, we observed an upregulation of total NR2B in the CIPN condition, which is consistent with at least some reports in paclitaxel models.[32] One possible interpretation is that chronic CIPN may involve broader alterations in NR2B expression, potentially including extrasynaptic pools. Extrasynaptic NR2B has been proposed to support sustained pathological signaling through tonic activation by ambient glutamate under chronic conditions.[33,34] While our study does not directly resolve synaptic versus extrasynaptic localization, the reduction of glutamate content and NR2B-related measures after WAA, together with the reversal by NMDAR activation, is compatible with a model in which WAA reduces the overall "gain" of NMDAR-related signaling in the dorsal horn.

VGluT2 provides another mechanistic bridge between presynaptic glutamate packaging/release and postsynaptic NMDAR activation. Increased VGluT2 expression has been implicated in chronic pain states and may reflect enhanced capacity for glutamate vesicular loading and release.[35] In paclitaxel-induced CIPN, presynaptic input mediated by NMDARs in VGluT2-expressing excitatory neurons has been reported to be preferentially enhanced compared with inhibitory neurons, contributing to an excitation–inhibition imbalance within the dorsal horn.[36] Our VGluT2 neuron–specific α7nAChR deletion

data are particularly informative in this regard: when α7nAChR was removed from VGluT2$^+$ excitatory neurons, WAA lost much of its ability to reduce dorsal horn glutamate and to downregulate NR2B/VGluT2-related measures and downstream markers. This supports the interpretation that α7nAChR signaling within excitatory glutamatergic neurons plays an important role in linking WAA stimulation to reduced glutamatergic/NMDAR pathway activity. α7nAChR may influence presynaptic release probability, excitatory neuron excitability, or intracellular signaling cascades that shape NR2B-related plasticity.

α7nAChR has been implicated broadly in neurological disorders, including neuropathic pain.[37,38] Prior studies indicate that α7nAChR is predominantly expressed in neurons within the dorsal root ganglia, spinal cord, and brain,[39–41] and α7nAChR agonists have shown analgesic potential in chemotherapy models without compromising antitumor efficacy.[11] Additional work suggests that α7nAChR activation can reduce chronic pain through anti-inflammatory mechanisms involving microglia in some settings,[42] and α7nAChR signaling has been linked to spinal mechanisms in related pain conditions such as chemotherapy-induced bone pain.[21] Our study extends this literature by providing convergent evidence supporting a primarily neuronal mechanism in the spinal dorsal horn, particularly within VGluT2-expressing excitatory neurons, as a key contributor to WAA-mediated analgesia in paclitaxel-induced CIPN.

Acupuncture-based interventions are increasingly used as supportive care for chemotherapy-related symptoms, including pain.[43] Consistently, a systematic review and meta-analysis found that wrist-ankle acupuncture (WAA) improves pain outcomes across several chronic pain conditions and may be safer than oral medication.[44] WAA may offer pragmatic advantages compared with some traditional techniques, including relatively shallow needling and reduced reliance on strong "Deqi" sensations, which could improve patient acceptability and standardization in supportive care settings.[45] Our findings provide mechanistic support for the clinical rationale of WAA in paclitaxel-induced neuropathic pain by implicating spinal neuronal α7nAChR and glutamatergic/NMDAR-related signaling as key nodes that can be modulated in parallel with behavioral benefit.

Despite these insights, several limitations should be acknowledged. First, our mechanistic analysis focused on the spinal dorsal horn, and potential contributions of α7nAChR signaling in other pain-processing regions (eg., dorsal root ganglia and supraspinal circuits) were not examined. Defining how spinal mechanisms interface with broader nociceptive circuitry will be important for understanding the full neurobiological basis of WAA-induced analgesia. Second, although we quantified glutamate levels and multiple molecular readouts (NR2B, VGluT2, p-JNK, p-CREB, CGRP, and c-fos), we did not directly assess synaptic physiology or neuronal firing. Future studies incorporating electrophysiological recordings or calcium imaging would help link these biochemical changes to functional alterations in dorsal horn circuit activity.

Conclusion

In summary, our study supports a model in which WAA alleviates paclitaxel-induced CIPN by engaging spinal neuronal α7nAChR, thereby modulating glutamatergic/NMDAR-related signaling and associated downstream markers within the dorsal horn. These findings provide mechanistic insight into how WAA may reduce CIPN hypersensitivity and highlight neuronal α7nAChR—particularly within VGluT2-expressing excitatory neurons—as a critical component of this analgesic pathway.

Data Sharing Statement

The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request. Requests should be directed to Zhifu Wang (2007015@fjtcm.edu.cn) or Xiangmei Yu (yxmtcm@163.com).

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Disclosure

The authors declare that they have no competing interests in this work.

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