Oral Application of Magnesium-L-Threonate Attenuates Vincristine-induced Allodynia and Hyperalgesia by Normalization of Tumor Necrosis Factor-α/Nuclear Factor-κB Signaling

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ABSTRACT

Background: Antineoplastic agents, including vincristine, often induce neuropathic pain and magnesium deficiency clinically, but the causal link between them has not been determined. No drug is available for treating this form of neuropathic pain.

Methods: Injection of vincristine (0.1 mg · kg^{-1} · day^{-1}, intraperitoneally, for 10 days) was used to induce nociceptive sensitization, which was accessed with von Frey hairs and the plantar tester in adult male Sprague–Dawley rats. Magnesium-L-threonate was administered through drinking water (604 mg · kg^{-1} · day^{-1}). Extracellular and intracellular free Mg^{2+} were measured by Calmagite chromometry and flow cytometry. Molecular biologic and electrophysiologic experiments were performed to expose the underlying mechanisms.

Results: Vincristine injection induced allodynia and hyperalgesia (n = 12), activated tumor necrosis factor-α/nuclear factor-κB signaling, and reduced free Mg^{2+} in cerebrospinal fluid by 21.7 ± 6.3% (mean ± SD; n = 13) and in dorsal root ganglion neurons by 27 ± 6% (n = 11). Reducing Mg^{2+} activated tumor necrosis factor-α/nuclear factor-κB signaling in cultured dorsal root ganglion neurons. Oral application of magnesium-L-threonate prevented magnesium deficiency and attenuated both activation of tumor necrosis factor-α/nuclear factor-κB signaling and nociceptive sensitization (n = 12). Mechanistically, vincristine induced long-term potentiation at C-fiber synapses, up-regulated *N*-methyl-D-aspartate receptor type 2B subunit of *N*-methyl-D-aspartate receptor, and led to peptidergic C-fiber sprouting in spinal dorsal horn (n = 6 each). The vincristine-induced pathologic plasticity was blocked by intrathecal injection of nuclear factor-κB inhibitor (n = 6), mimicked by tumor necrosis factor-α, and substantially prevented by oral magnesium-L-threonate (n = 5).

Conclusions: Vincristine may activate tumor necrosis factor-α/nuclear factor-κB pathway by reduction of intracellular magnesium, leading to spinal pathologic plasticity and nociceptive sensitization. Oral magnesium-L-threonate that prevents the magnesium deficiency is a novel approach to prevent neuropathic pain induced by chemotherapy. **(ANESTHESIOLOGY 2017; 126:1151-68)**

THE neuropathic pain produced by anticancer agents, also called painful chemotherapy-induced peripheral neuropathy (CIPN), is a serious side effect, occurring in 30 to 40% of patients.[1] CIPN can be extremely painful, leading to dose reduction or even discontinuation of chemotherapy.[2] After termination of chemotherapy, the CIPN is only partly resolved in many patients and even not reversible at all in some cases,[3,4] causing significant loss of functional abilities and decrease in life quality of cancer survivors.[5] At present, no effective drug for prevention or treatment of CIPN is available.

What We Already Know about This Topic

- Neuropathic pain induced by chemotherapy is accompanied by magnesium deficiency and activation of tumor necrosis factor-α/nuclear factor-κB signaling

What This Article Tells Us That Is New

- Vincristine-induced allodynia and hyperalgesia are reduced by oral magnesium-L-threonate administration
- Oral magnesium-L-threonate administration also blocked tumor necrosis factor-α/nuclear factor-κB signaling and spinal cord neuroplasticity after vincristine administration

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Previous studies have shown that many anticancer agents, including cisplatin,[6] paclitaxel,[7] and epidermal growth factor receptor–targeting antibodies (cetuximab),[8] cause both hypomagnesemia and neuropathic pain in human patients. Accordingly, supplementation of Mg^{2+} with intravenous infusion of calcium gluconate and magnesium sulfate has been tested to treat CIPN clinically. The outcomes, however, are controversial, with some studies reporting that the therapy is effective for prevention of oxaliplatin-induced CIPN,[1,9] while others show no effect.[10,11] Previous studies show that increasing plasma Mg^{2+} three-fold *via* intravenous infusion of magnesium sulfate is incapable of elevating Mg^{2+} levels in the central nervous system.[12,13] This may explain the negative outcome with calcium–magnesium infusion at least in some patients. Recently, we developed a novel magnesium compound (magnesium-L-threonate [L-TAMS], formerly MgT) that can increase brain Mg^{2+} *via* chronic oral application. Oral L-TAMS is capable of improving the memory function in naive rats[14] and in a transgenic mouse model of Alzheimer disease[15] and of preventing and restoring the short-memory deficits produced by peripheral nerve injury.[16] Recent human clinical trials show that L-TAMS treatment improves global cognitive abilities of older adults.[17] However, whether oral L-TAMS is able to prevent CIPN remains elusive.

It has been well established that proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), play a key role for the initiation of nociceptive sensitization induced by both peripheral nerve injury[18–20] and chemotherapeutic drugs.[21–23] Nuclear factor-κB (NF-κB), a transcription factor for many cytokines including TNF-α, has been proved important for generation of nociceptive sensitization induced by both peripheral nerve injury[24,25] and paclitaxel.[26] It has long been demonstrated that magnesium deficiency is associated with elevation of serum proinflammatory cytokines including TNF-α in human patients[27] and in animals[28] and is sufficient to induce hyperalgesia (an increased response to noxious stimuli).[29,30] Whereas whether magnesium deficiency activates TNF-α/NF-κB signaling in the nervous system has not been investigated.

At present, whether vincristine-induced peripheral neuropathy[31,32] is also associated with magnesium deficiency is unknown. The causal link between magnesium deficiency and CIPN has not been determined. The objective of the study was to address the questions and to test if oral application of L-TAMS that can increase brain Mg^{2+} is able to prevent vincristine-induced nociceptive sensitization. We found that vincristine injection reduced intracellular free Mg^{2+} and activated TNF-α/NF-κB signaling in dorsal root ganglion (DRG) neurons. Importantly, we showed that reduction of Mg^{2+} activated TNF-α/NF-κB signaling in cultured DRG neurons. Oral application of L-TAMS that prevented magnesium deficiency substantially attenuated the molecular and behavioral abnormalities induced by vincristine.

Materials and Methods

Animals

Male Sprague–Dawley rats (220 to 250 g) were purchased from the Institute of Experimental Animals of Sun Yat-Sen University, Guangzhou, China. The rats were housed (one rat per cage) in a temperature-controlled room and permitted free access to sterile water and standard laboratory chow. All animal experimental procedures were approved by the animal care and use committee of our university and carried out in accordance with the guidelines of the National Institutes of Health (Beijing, China) on animal care and ethical guidelines. All animals were randomly assigned to different experimental or control conditions.

Drug Administration

Vincristine sulfate (Main Luck Pharmaceuticals Inc., China) dissolved in saline to a concentration of 50 μg/ml was intraperitoneally injected at a dose of 0.1 mg/kg daily for 10 consecutive days.[33,34] Control animals received an equivalent volume of saline. According to previous studies,[14,16] L-TAMS (NEUROCENTRIA Inc., USA) was administered *via* drinking water ($604\ mg \cdot kg^{-1} \cdot day^{-1}$) with $50\ mg \cdot kg^{1} \cdot day^{-1}$ elemental magnesium, initiated 2 days before the first dose of vincristine and maintained until the end of the experiments. The average drinking water (~30 ml/day) and daily food intake that contained 0.15% elemental Mg^{2+} were monitored. The concentration of L-TAMS in the drinking water was adjusted based on these parameters to reach the target dose.

Behavioral Tests

Animals were habituated, and basal nociceptive sensitivity was tested before drug administration. The mechanical withdrawal threshold was assessed with the up-down method described previously,[35] using a set of von Frey hairs with logarithmically incremental stiffness from 0.41 to 26.0 *g* (0.41, 0.70, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14, and 26.0 *g*). Briefly, the 2.04 *g* stimulus, in the middle of the series, was applied first. In the event of paw withdrawal absence, the next stronger stimulus was chosen. Otherwise, a weaker stimulus was applied. Each stimulus consisted of a 6- to 8-s application of the von Frey hair to the sciatic innervation area of the hind paws with 5-min intervals. The quick withdrawal or licking of the paw in response to the stimulus was considered a positive response.

Thermal hypersensitivity was measured using a plantar test (7370; UgoBasile, Italy), according to the method described previously.[36] Briefly, a radiant heat source beneath a glass floor was aimed at the adequate plantar surface of the hind paw. Three measurements of withdrawal latency and withdrawal duration were recorded for each hind paw, with a minimal value of 0.5 s and a maximum of 25 s. The hind paw was tested alternately with greater than 8-min intervals between consecutive tests.

The experimenter who conducted the behavioral tests was blinded to all treatments. Around 10% of rats that did not show mechanical allodynia after vincristine injection were excluded from further analysis.

Culture of DRG Neurons

DRG neurons were dissociated using enzyme digestion as described previously.[37] Briefly, ganglia from the cervical to the lumbar level from every rat were excised, freed from their connective tissue sheaths, and broken into pieces with a pair of sclerotic scissors, in DMEM/F12 medium (GIBCO, USA) under low temperature (in a mixture of ice and water). After enzymatic and mechanical dissociation, DRG neurons were collected for flow cytometry analysis or seeded on a 24-well plate coated with poly-L-lysine (Sigma, USA) in a humidified atmosphere (5% CO_2, 37°C) overnight and then used for immunocytochemical analysis and Western blot.

Measurement of Extracellular and Intracellular Free Magnesium

To determine the free Mg^{2+} concentration in serum and cerebrospinal fluid (CSF) of rats, blood samples collected from the orbital sinus were centrifuged at 3,000 rpm for 10 min to isolate serum and CSF was collected from foramen magnum. The concentrations of free Mg^{2+} in the fluids were measured with calmagite chromometry method.[38] The intracellular Mg^{2+} concentration in DRG neurons was determined by flow cytometry (EPICS XL-MCL; Beckman Coulter, USA) using Magnesium Green (Invitrogen, Massachusetts, USA). The mean fluorescence intensity was used as an indicator of the free Mg^{2+} level in the neurons. The presented data represent the standard mean fluorescence intensity of 30,000 neurons in each rat.

Electrophysiologic Recording

C-fiber–evoked field potentials in the spinal dorsal horn were recorded, according to our method described previously.[39] Briefly, after electrical stimulation of the sciatic nerve, field potentials were recorded in the spinal dorsal horn (L4 and L5 segments) at the depth of 50 to 500 μm from the dorsal surface with a glass microelectrode, which was driven by an electronically controlled microstepping motor (Narishige Scientific Instrument Laboratory, Japan). The spinal field potentials were recorded and analyzed by the LTP program (http://www.ltp-program.com). Single square pulses (0.5-ms duration, in 1-min intervals) delivered to the sciatic nerve were used as test stimuli. The strength of stimulation was adjusted to 1.5 to 2 times the threshold for C-fiber response. The distance from the stimulation site at the sciatic nerve to the recording site in the lumbar spinal dorsal horn was around 11 cm. Only one experiment was conducted on each animal.

Western Blot

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and L4-L6 DRGs and the dorsal half of the spinal cord on bilateral sides were harvested. The samples were frozen at −80°C until used. Samples were homogenized on ice in 15 mmol/l Tris buffer containing a cocktail of proteinase inhibitors and phosphatase inhibitors. Protein samples were separated by gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The blots were placed in block buffer for 1 h at room temperature and incubated with the primary antibody against TNF-α (1:500; Santa Cruz, USA; mouse), NF-κB p65 (1:1,000; Abcam, United Kingdom; rabbit), phosphorylated NF-κB p65 (Ser311; 1:1,000; Abcam; rabbit), IκB-α (1:500; Abcam; rabbit) overnight at 4°C. The blots were then incubated with horseradish peroxidase–conjugated immunoglobulin G. Enhanced chemiluminescent liquid (Pierce, USA) was used to detect the immune complex. The band was quantified with a computer-assisted imaging analysis system (ImageJ; National Institutes of Health, USA).

Immunohistochemistry

Immunochemistry was performed as we described previously.[40] Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and perfused through the ascending aorta with 4% paraformaldehyde. The lumbar spinal cord segments and DRGs were removed and postfixed in the same fixative overnight. Cryostat sections (16 μm) were cut and processed for immunohistochemistry with the primary antibody for TNF-α (goat; 1:200; Invitrogen, USA), phosphorylated NF-κB p65 (Ser311; rabbit; 1:200; Abcam), NeuN (mouse; 1:400; Chemicon, USA), calcitonin gene–related peptide (CGRP; rabbit; 1: 200; CST, USA), plant lectin B4 (IB4; mouse; 1:100; Abcam), NF-200 (mouse; 1:200; Abcam), glial fibrillary acidic protein (GFAP; mouse; 1:500; Chemicon), and OX-42 (mouse; 1:200; Chemicon). After incubation overnight at 4°C, the sections were incubated with Cy3-conjugated and Alex-conjugated secondary antibodies for 1 h at room temperature. The stained sections were then examined and captured with a Leica fluorescence microscope (Leica DFC350 FX camera; Oskar Barnack, Germany) or LSM 780 confocal microscope (Zeiss, Germany). For quantification of CGRP, IB4, and NF-200 expression in the spinal dorsal horn, the fluorescence intensity of each area was analyzed with Image-Pro Plus 6.0 (Media Cybernetics, USA).

As NF-κB is weakly activated in physiologic conditions and low intensity of *p*-p65, staining is detected in the nucleus of DRG neurons and dorsal horn cells in the vehicle group. For analysis of the change in nuclear translocation of *p*-p65 in DRG and dorsal horn cells in different groups, the mean integral optical density value of *p*-p65 in six sections from three vehicle-treated rats (50 nuclei per section of DRG and 100 nuclei per section of dorsal horn cells) was measured by Image-Pro Plus 6.0 and served as baseline (100%). The percentage changes in other groups were calculated according to the baseline.

Statistical Analysis

All data were expressed as mean ± SD and analyzed using SPSS 13.0 (SPSS, USA). Student's *t* test was used for

comparison of protein expression level between two groups. The results from the behavioral tests, immunohistochemistry, electrophysiology, and flow cytometry were statistically analyzed with a one-way or two-way (repeated-measures) ANOVA followed by Tukey *post hoc* test. Correlation analysis was used to reveal the relationship between paw withdrawal thresholds (PWTs) and magnesium concentrations, and the Pearson product–moment coefficient was calculated. All data were normally distributed, the variance was homogeneous, and all probability values were two tailed. $P < 0.05$ was considered statistically significant. While no power analysis was performed, the sample size was determined according to our and peers' previous publications on painful behavior and pertinent molecular studies.

Results

Chronic Oral Application of L-TAMS Prevents Magnesium Deficiency, Mechanical Allodynia, and Thermal Hyperalgesia Induced by Vincristine

To investigate the effects of chronic oral application of L-TAMS on the nociceptive sensitization induced by vincristine and the underlying mechanisms, rats were divided randomly into the following four groups: the vincristine group receiving an intraperitoneal injection of vincristine ($0.1\,mg \cdot kg^{-1} \cdot day^{-1}$ for 10 consecutive days) and drinking normal water; the vincristine + L-TAMS group receiving L-TAMS (604 mg/kg, daily) in drinking water 2 days before vincristine injection and until the end of the experiments; the L-TAMS group receiving L-TAMS and a saline injection; and the vehicle group drinking normal water and receiving a saline injection.

We found that both PWTs and paw withdrawal latencies reduced significantly in the vincristine group, starting at day 5 but not at day 2 after vincristine injection and persisting for at least 20 days, indicating that chronic mechanical allodynia and thermal hyperalgesia were produced. Interestingly, oral application of L-TAMS significantly attenuated the vincristine-induced mechanical allodynia and thermal hyperalgesia but did not affect PWTs and paw withdrawal latencies in the vehicle-treated rats (fig. 1, A and B). To determine whether the vincristine-induced behavioral changes are associated with magnesium deficiency, free Mg^{2+} concentrations in serum, CSF, and intracellular Mg^{2+} level in DRG neurons were measured 2, 5, and 10 days after vincristine or vehicle injection. Compared to the vehicle group, free Mg^{2+} concentrations in serum, in CSF, and in DRG neurons were significantly reduced in the vincristine group at day 5 and day 10 but not at day 2 after vincristine injection, which is in parallel with the onset of vincristine-induced mechanical allodynia and thermal hyperalgesia. As shown in fig. 1, C–E, at 10 days after vincristine injection, Mg^{2+} in serum was reduced by 22.6 ± 6.9% (from 0.81 ± 0.16 to 0.63 ± 0.20 mM), in CSF by 21.7 ± 6.3% (from 0.83 ± 0.15 to 0.65 ± 0.18 mM), and in intracellular Mg^{2+} of DRG neurons by 27 ± 6%. No difference between the vehicle and the vincristine + L-TAMS groups at any time point was detected. The free Mg^{2+} level in the L-TAMS group was also not different from that in the vehicle group. The results indicate that vincristine induces the extracellular and intracellular magnesium deficiency and that oral application of L-TAMS prevents the magnesium deficiency induced by vincristine but does not affect the Mg^{2+} level in the vehicle-treated rats. Correlation analysis revealed that PWTs were positively related to the free Mg^{2+} levels in CSF and in DRG neurons, but not to serum 10 days after onset of vincristine (Supplemental Digital Content 1, Figure, http://links.lww.com/ALN/B390, showing the correlation analysis of free Mg^{2+} and PWT), suggesting that magnesium deficiency in CSF and in DRG neurons is associated with vincristine-induced nociceptive sensitization.

Vincristine Treatment Induces LTP at C-fiber Synapses, Up-regulation of N-methyl-D-aspartate Receptor Type 2B (NR2B) Subunit of N-methyl-D-aspartate (NMDA) Receptor, and Sprouting of Peptidergic C-fibers in the Spinal Dorsal Horn, and the Effects Are Attenuated by Oral L-TAMS

At present, how could vincristine lead to severe pain in human patients is poorly understood. As afferent C-fibers that conduct nociceptive signals to the spinal cord make the first synapses with dorsal horn neurons,[41] we compared the strength of synaptic transmission mediated by C-fibers by recording spinal C-fiber–evoked field potentials in the four groups of rats 7 to 10 days after injection of vincristine or vehicle with or without L-TAMS application. As shown in figure 2A, the amplitudes of the field potentials enhanced with increasing stimulation intensity in each group, but the magnitude of the change was substantially different. Compared with the vehicle group, a significant upright shift of the stimulus–response curve was evident in the vincristine group, indicating that the synaptic transmission mediated by afferent C-fibers is increased in the vincristine group. The shift was significantly smaller in the vincristine + L-TAMS group than that in vincristine group. There was no difference between the vehicle and the L-TAMS groups. The results indicate that the treatment with vincristine induces LTP at C-fiber synapses, and oral application of L-TAMS attenuates the vincristine-induced LTP but does not affect basal synaptic transmission mediated by C-fibers.

Activation of NMDA receptor (NMDAR) is critically involved in LTP in both the hippocampus and the spinal dorsal horn.[42] A previous study shows that up-regulation of NR2B but not of NR2A subunit of NMDAR in the dorsal horn is critically involved in thermal hyperalgesia produced by partial chronic constriction injury of the sciatic nerve.[43] We therefore examined whether vincristine injections also affect NMDAR expression 10 days after vincristine and vehicle injection. As shown in figure 2, B and C, the protein levels of NR2B were significantly lower in the hippocampus (by 59 ± 19%) and higher in the spinal dorsal horn (by 185 ± 34%) in the vincristine group than in the vehicle group. NR2B expression in the L-TAMS and the vincristine + L-TAMS

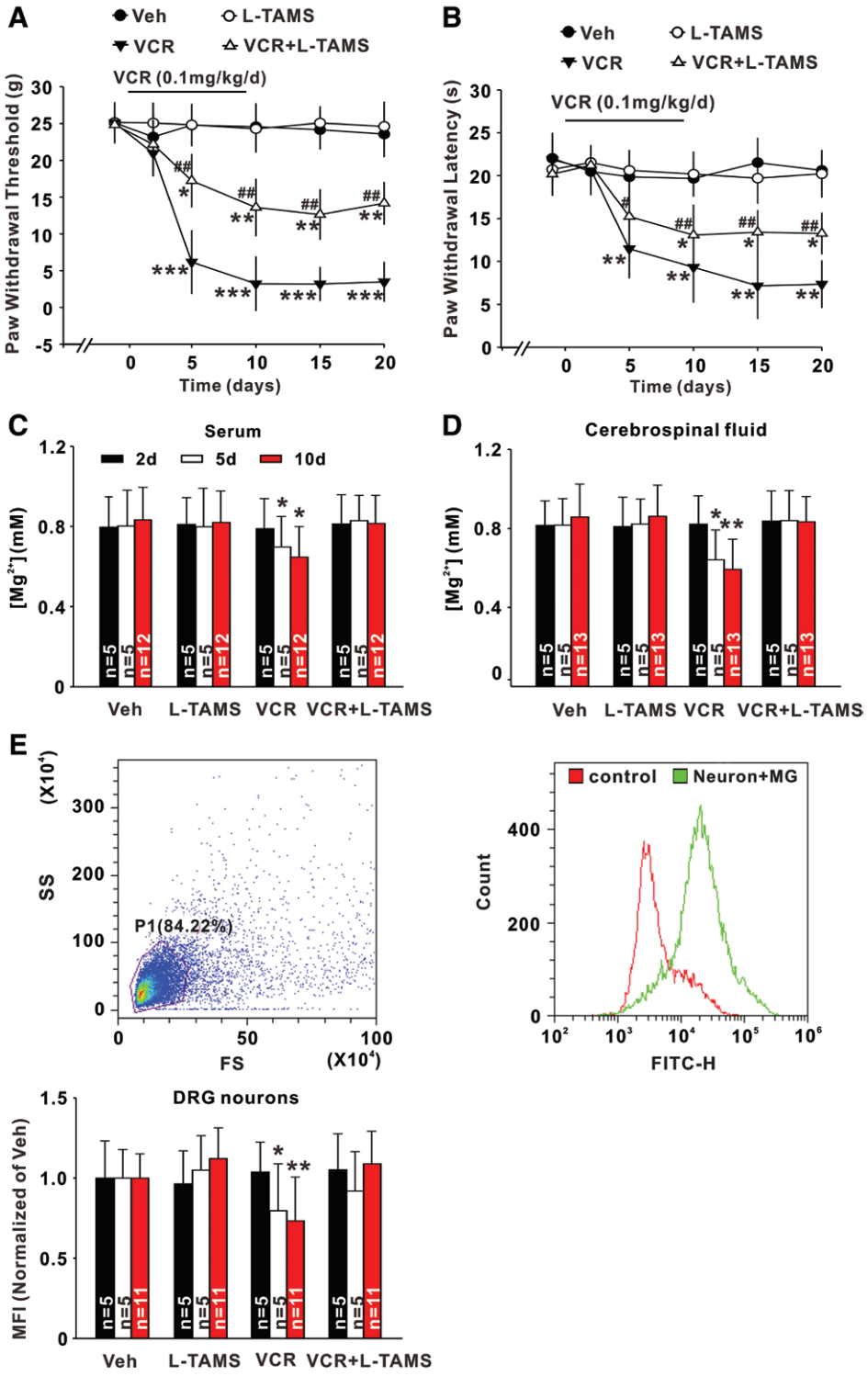

Fig. 1. Magnesium-L-threonate (L-TAMS) attenuates the mechanical allodynia and thermal hyperalgesia and prevents magnesium deficiency induced by vincristine (VCR). The time courses of paw withdrawal threshold (*A*) and paw withdrawal latency (*B*) in vehicle (Veh), L-TAMS, VCR, and L-TAMS + VCR groups are shown. Treatment with L-TAMS significantly suppressed VCR-induced mechanical allodynia and thermal hyperalgesia (n = 12 per group). (*C*, *D*) Free Mg^{2+} concentrations in serum and in cerebrospinal fluid of different groups measured with magnesium green (MG) at different time point are shown. The number of animals used in each group is indicated in the histograms. (*E*) Representative flow physical diagram and histograms show the level of intracellular free magnesium of dorsal root ganglion (DRG) neurons (assessed as mean fluorescence intensity [MFI] of 30,000 cells per rat). The number of animals used in each group is indicated in the histograms. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the Veh group; $\#P < 0.05$, $\#\#P < 0.01$, compared with the VCR group. FITC-H = fluorescein isothiocyanare-high; FS = forward scatter; SS = side scatter.

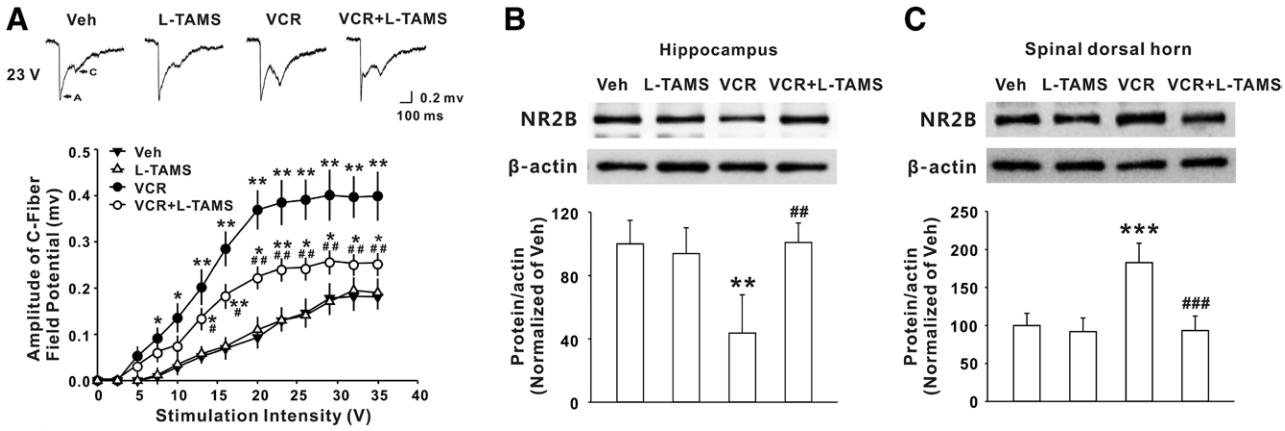

Fig. 2. Oral magnesium-L-threonate (L-TAMS) attenuates the long-term potentiation at spinal C-fiber synapses and the up-regulation *N*-methyl-D-aspartate receptor type 2B (NR2B) subunit of *N*-methyl-D-aspartate receptor produced by vincristine. (*A*) The stimulus–response curves of C-fiber–evoked field potentials in the vehicle (Veh), L-TAMS, vincristine (VCR), and L-TAMS + VCR groups are shown (n = 6 per group); the traces show the representative original recordings of the field potentials in different groups. (*B*, *C*) The Western blots show NR2B levels in different groups 10 days after VCR and Veh injection (n = 6 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the Veh group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, compared with the VCR group. A and C = A-fiber and C-fiber responses, respectively.

groups was not different from that in the vehicle group. The data demonstrate that vincristine differentially regulates NR2B expression in the hippocampus and in the spinal dorsal horn, and chronic oral application of L-TAMS blocks the region-dependent effect but does not affect the NR2B expression in the rats receiving vehicle injection. The up-regulation of NR2B in the dorsal horn may count for the vincristine-induced spinal LTP.

To explore the morphologic basis of the vincristine-induced long-lasting LTP at C-fiber synapses, we analyzed CGRP-positive peptidergic C-fibers, IB4-positive nonpeptidergic C-fibers, and NF-200–positive A-fibers in the spinal dorsal horn of the four groups of rats. Consistent with previous studies,[44] CGRP-positive fibers were observed mainly in lamina I and outer lamina II in the vehicle group (fig. 3Aa), while in the vincristine group, they were also observed in deep lamina (fig. 3Ac). The CGRP-positive area in lamina I to II increased by 222 ± 36% and in lamina III to IV by 275 ± 27% in the vincristine group compared to those in the vehicle group (fig. 3Aa, c, e, and f). Similar to its effect on C-fiber–evoked field potentials, chronic oral application of L-TAMS reduced but not abolished the morphologic change, as the CGRP-positive area in the vincristine + L-TAMS group was significantly smaller than that in the vincristine group but was still larger compared to the vehicle group (Fig. 3Aa and c–f). No difference in CGRP expression was detected between the vehicle and the L-TAMS groups (fig. 3Aa, b, e, f, and C). There was no difference in IB4-positive and NF-200–positive areas among the groups (fig. 3Ba–j). Consistently, Western blot showed that the expression of CGRP but not NF-200 in the spinal dorsal horn was enhanced in the vincristine-treated rats, and the up-regulation of CGRP was again reduced but not abolished by oral L-TAMS (fig. 3C). Data demonstrate that vincristine induces a morphologic plasticity at spinal C-fiber synapses, which is substantially prevented by chronic oral application of L-TAMS.

Chronic Oral Application of L-TAMS Prevents Vincristine-induced Up-regulation of TNF-α in Both DRG and Spinal Dorsal Horn

Accumulating evidence has demonstrated that TNF-α plays a key role in nociceptive sensitization induced by peripheral nerve injury,[18] by paclitaxel in rats,[45] and by oxaliplatin in cancer patients.[46] To test if TNF-α is also involved in the vincristine-induced nociceptive sensitization, we measured the protein levels of TNF-α in DRG and in spinal dorsal horn tissues with Western blot at different time points after the onset of vincristine injection (figs. 4 and 5). As shown in figures 4A and 5A, the significant increase in TNF-α was evident on day 3 and peaked on day 10 after the onset of vincristine treatment, and the change persisted for at least 10 days after the termination of vincristine injection (20 days after injection). To test whether L-TAMS may affect TNF-α overexpression, we analyzed the cytokine expression in DRG and spinal dorsal horn tissues from the four groups of rats that had been treated with saline or vincristine with or without L-TAMS for 10 days. The levels of TNF-α in both the DRG (fig. 4B) and the spinal dorsal horn (fig. 5B) in the vincristine + L-TAMS group were significantly lower than those in the vincristine group and were not different from those in the vehicle group. No difference between the L-TAMS group and the vehicle group was detected. The data indicate that oral L-TAMS prevents the vincristine-induced overproduction of TNF-α but does not affect its basal expression.

Double-staining performed with DRGs harvested 10 days after vincristine or vehicle injection showed that TNF-α was colocalized with NF-200 (a marker for A-type neuron), IB4

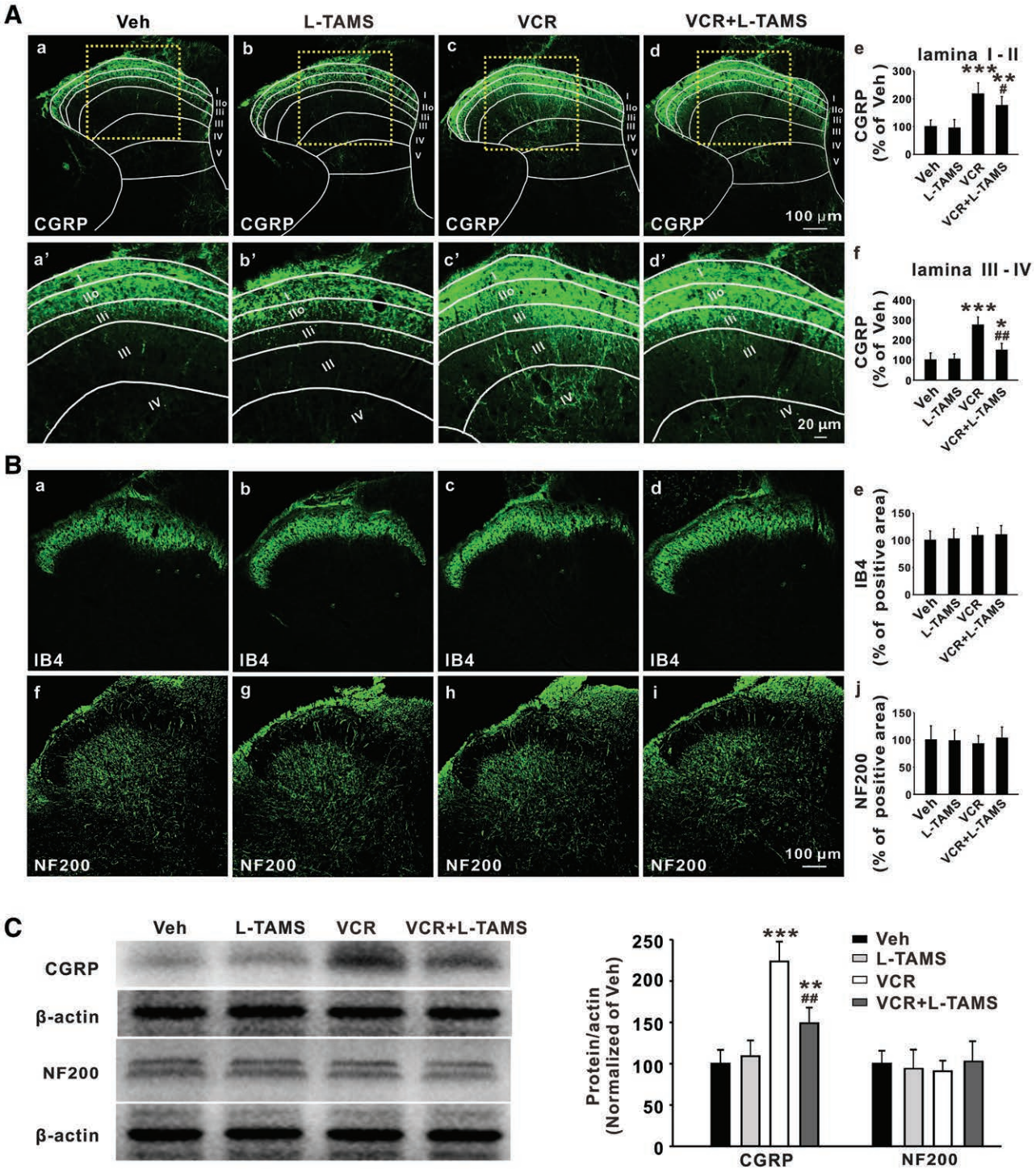

Fig. 3. Vincristine injection induces calcitonin gene–related peptide (CGRP)–positive C-fiber sprouting in the dorsal horn, and the morphologic change is attenuated by oral magnesium-L-threonate (L-TAMS). (*A*) The representative photographs show the expression of CGRP (a marker for peptidergic C-fiber) in the spinal dorsal horn in different groups of rats, and the histograms show summary data of CGRP expression in lamina I to II and lamina III to IV (n = 5 per group). The amplifying figures marked by *dashed lines* in *a–d* are shown in *a′–d′*. (*B*) No difference in expression of IB4 (a marker for nonpeptidergic C-fiber) and NF-200 (a marker for A-fiber) was detected among the groups (n = 4 per group). (*C*) The Western blots show the expression of CGRP and NF-200 in the spinal dorsal horn in different groups, as indicated (n = 5 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the vehicle (Veh) group; #*P* < 0.05, ##*P* < 0.01, compared with the vincristine (VCR) group.

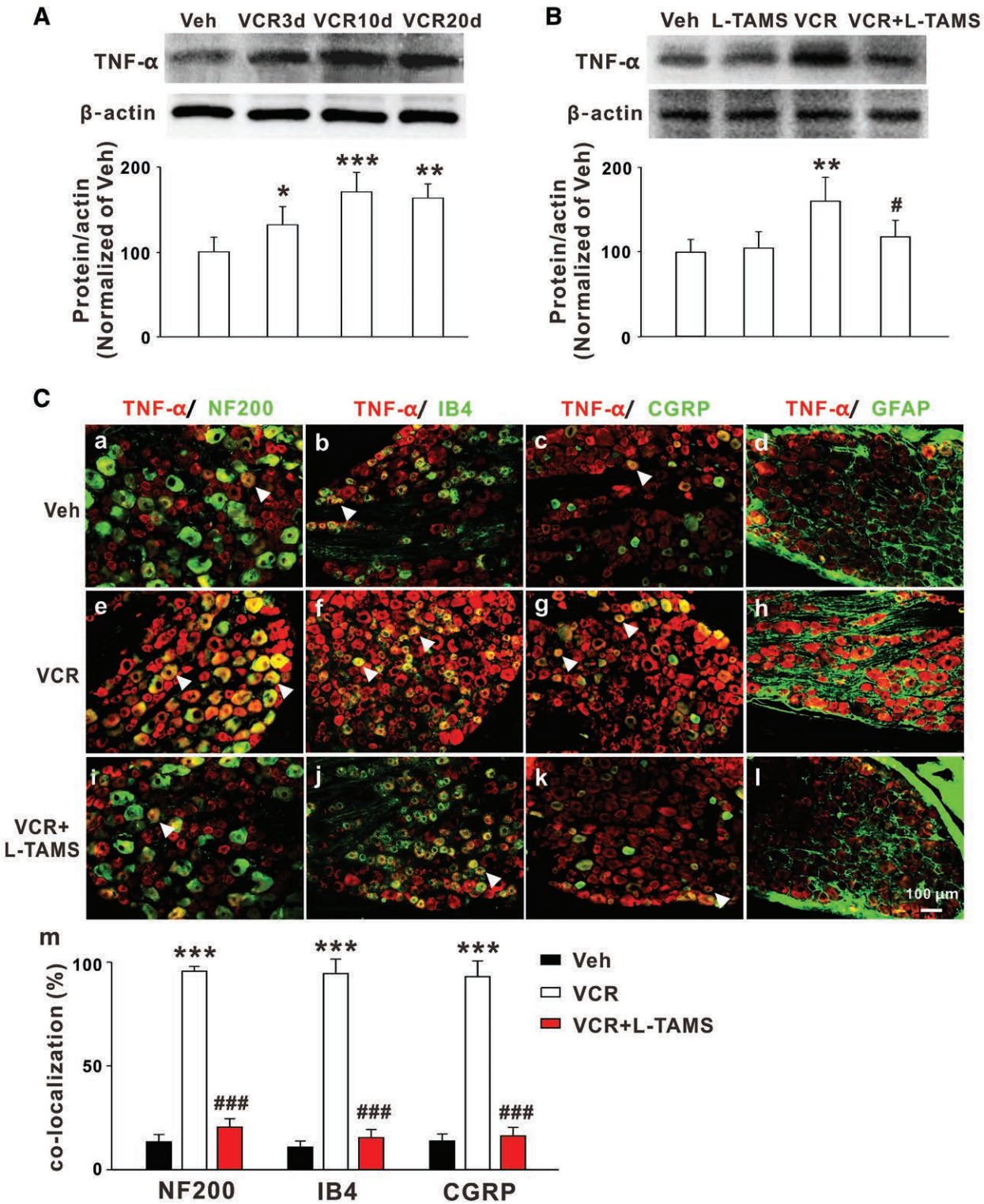

Fig. 4. Oral application of magnesium-L-threonate (L-TAMS) blocks the vincristine (VCR)-induced up-regulation of tumor necrosis factor-α (TNF-α) in dorsal root ganglion neurons (DRG). (*A*) The Western blots show the levels of TNF-α in DRG tissue at different time points after the onset of vincristine (VCR) injection (n = 6 per group). (*B*) The VCR-induced TNF-α up-regulation was completely blocked by coadministration of L-TAMS (n = 6 per group). (*C*) The photographs of the double immunofluorescence staining show that TNF-α is only colocalized with neuron markers (NF-200, IB4, and calcitonin gene–related peptide [CGRP]) but not with satellite glial cell marker (glial fibrillary acidic protein [GFAP]). The *arrowheads* indicate the coimmunostaining of TNF-α and neuron markers. VCR injection increased TNF-α expression in DRG neurons, which was blocked by oral L-TAMS (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the vehicle (Veh) group; #$P < 0.05$, ###$P < 0.001$, compared with the VCR group.

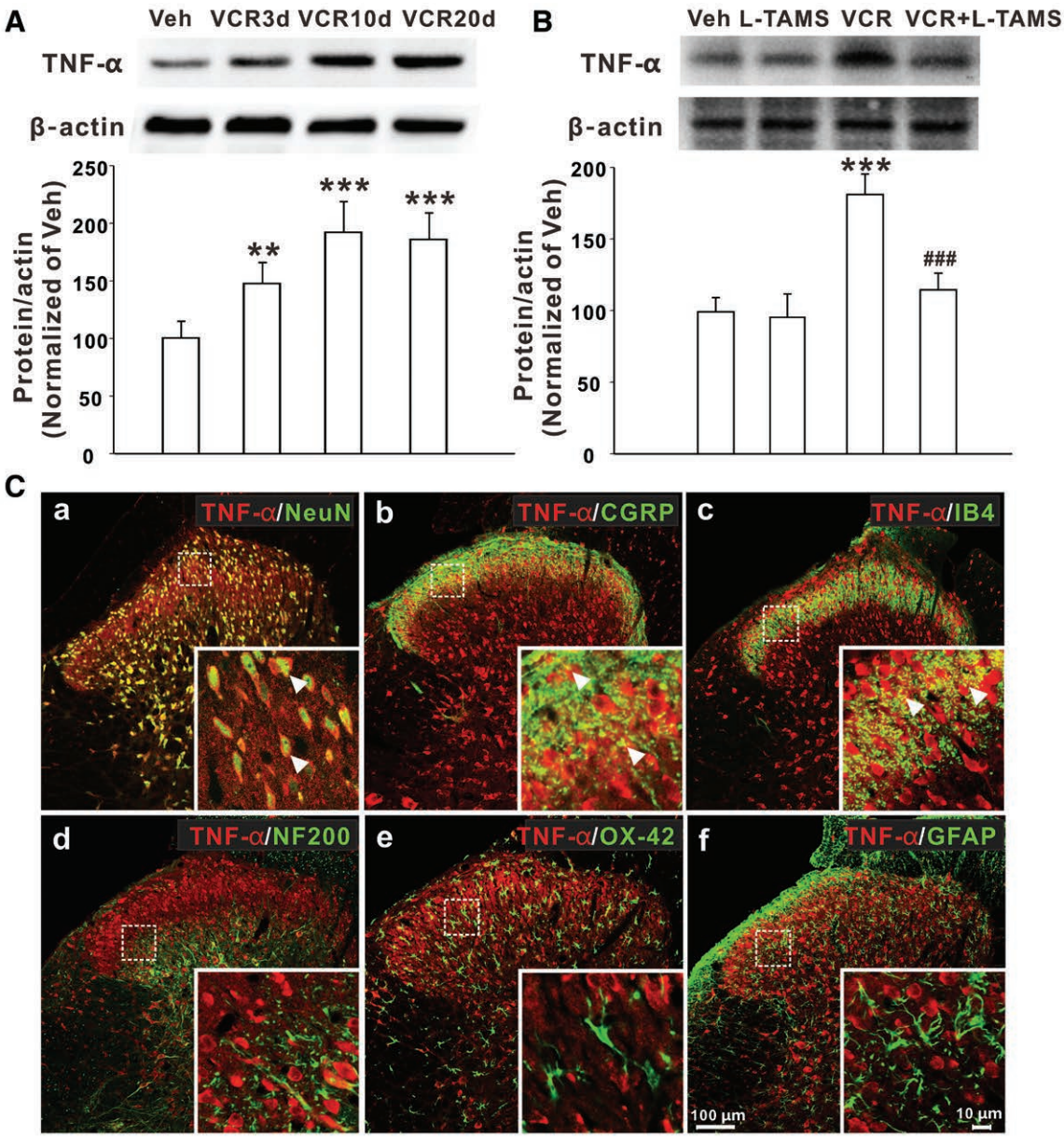

Fig. 5. Oral magnesium-L-threonate (L-TAMS) abolishes the vincristine (VCR)-induced up-regulation of tumor necrosis factor-α (TNF-α) in the spinal dorsal horn. (*A*) The time course of TNF-α expression in the spinal dorsal horn at different time points after the onset of vincristine (VCR) injection is shown (n = 6 per group). (*B*) The up-regulation of TNF-α by VCR was blocked by L-TAMS (n = 6 per group). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the vehicle (Veh) group; $###P < 0.001$, compared with the VCR group. (*C*) The double immunofluorescence staining with confocal microscopy shows that TNF-α is located in the nuclei of dorsal horn neurons (NeuN, a), C-fibers (IB4, b; and calcitonin gene–related peptide [CGRP], c) but not in A-fibers (NF200, d), microglia (OX-42, e), and astrocyte (glial fibrillary acidic protein [GFAP]; f). The *arrowheads* indicate the coimmunostaining of TNF-α and cell markers.

(a marker for nonpeptidergic C-type neuron), and CGRP (a marker for peptidergic C-type neuron), but not with GFAP (a marker for satellite glial cells) in the vehicle, the vincristine, and the vincristine + L-TAMS groups (fig. 4C). The data indicate that, in DRG, TNF-α is only expressed in neurons but not in satellite glial cells. In the vehicle group, around 13% of each type of DRG neurons expressed TNF-α (fig. 4Ca–c and m), while in the vincristine group, more than 92% of them did so (fig. 4Ce–g and m). The percentages of TNF-α–expressing neurons in the vincristine + L-TAMS group were not different from those in the vehicle group (fig. 4Cm). The data suggest that L-TAMS prevents vincristine-induced TNF-α up-regulation in all types of DRG neurons. In the spinal cord, TNF-α was expressed virtually in all dorsal horn neurons (colocalized with NeuN, a marker for nucleus of neuron), to a less extent in CGRP- and IB4-positive C-fibers (fig. 5Ca–c) 10 days after vincristine injection. TNF-α was not colocalized with NF-200 (a marker for A-fibers), OX-42 (a marker for microglia), and

GFAP (a marker for astrocyte; fig. 5Cd–f) 10 days after vincristine injection. The results suggest that the increased TNF-α in the cell bodies of C-type neurons but not of A-type neurons is transported to their central axons in the dorsal horn after vincristine injection. This is in line with previous studies that vincristine induces a significant decrease in axonal microtubules in myelinated fibers[47] but not in unmyelinated C-fibers.[48]

Chronic Oral Application of L-TAMS Attenuates the Vincristine-induced Activation of NF-κB in Both DRG and Spinal Dorsal Horn

NF-κB is critically involved in nociceptive sensitization,[25] and TNF-α leads to allodynia by promoting itself and other inflammatory cytokine production *via* activation of NF-κB.[49,50] It is well established that NF-κB p50/p65 is located in the cytoplasm and sequestered by binding to its inhibitor of NF-κB (IκB-α). TNF-α activates NF-κB by phosphorylation of p65 and IκB-α, and then phospho-IκB-α (*p*-IκB-α) is degenerated and *p*-p65 is translocated into the nucleus, where it activates a variety of target genes, including TNF-α.[25] Accordingly, to determine whether NF-κB is activated by vincristine, we measured total p65, *p*-p65, and IκB-α in DRG and in spinal dorsal horn tissues with Western blots and the nuclear expression of *p*-p65 with immunohistochemistry in four groups of rats (figs. 6 and 7). In the vincristine group, an increase of *p*-p65 and a decrease of IκB-α in both DRG and the spinal dorsal horn were evident on day 3 and day 10 after the onset of vincristine injection, and the changes persisted for at least 10 days after the end of vincristine injection (figs. 6A and 7A). While in the vincristine + L-TAMS group, the changes in *p*-p65 and IκB-α expression were significantly smaller compared to the vincristine group but were still larger compared to the vehicle group (figs. 6B and 7B), indicating that oral L-TAMS partially prevents activation of NF-κB induced by vincristine.

Double-staining revealed that in DRG, *p*-p65 was located in the nuclei of NF-200–positive A-type neurons and IB4-positive and CGRP-positive C-type neurons but not in the nuclei of satellite glial cells (fig. 6C). In the spinal dorsal horn, *p*-p65 was colocalized with NeuN (a marker for neuron nucleus), but not with OX-42 and GFAP (Figure, Supplemental Digital Content 2, http://links.lww.com/ALN/B391, showing the cell types that express *p*-p65 in the spinal dorsal horn in the three groups) in the vehicle, the vincristine, and the vincristine + L-TAMS groups. Furthermore, compared with the vehicle group, in the vincristine group, *p*-p65 in the nuclei of DRG neurons increased by 383 ± 48% and in the nuclei of the dorsal horn neurons by 196 ± 21% (figs. 6Da, c, e and 7Ca, c, e), indicating that *p*-p65 was translocated into the nuclei. Again, the nuclear expression of *p*-p65 was significantly lower in the vincristine + L-TAMS group than in the vincristine group (figs. 6Dc–e and 7Cc–e) but was still higher than in the vehicle group (figs. 6Da, d, e and 7Ca, d, e). No difference was detected between the vehicle and the L-TAMS groups (figs. 6Da, b, e and 7Ca, b, e). The data suggest that L-TAMS attenuates the activation of NF-κB induced by vincristine and does not affect its basal activity.

Magnesium Deficiency Activates TNF-α/NF-κB Signaling in Cultured DRG Neurons

At present, how could vincristine lead to the activation of TNF-α/NF-κB signaling is unknown. Our findings that supplement of Mg^{2+} by oral L-TAMS prevented intracellular magnesium deficiency of DRG neurons and attenuated the activation of TNF-α/NF-κB in DRG and in spinal dorsal horn induced by vincristine strongly suggested that vincristine may activate the signaling pathway by magnesium deficiency. To confirm this, we tested if TNF-α/NF-κB activity is regulated by extracellular Mg^{2+} in cultured DRG neurons. As injection of vincristine reduced free Mg^{2+} in CSF from around 0.8 to 0.6 mM (fig. 1D), extracellular Mg^{2+} at the two concentrations was tested. Western blots revealed that levels of TNF-α and *p*-p65 were significantly higher in the DRG neurons cultured with 0.6 mM Mg^{2+} than those cultured with 0.8 mM Mg^{2+} (fig. 8Aa and b). Immunohistochemistry showed that the level of *p*-p65 in nuclei increased by 223 ± 47% in neurons cultured with 0.6 mM Mg^{2+}, compared to those cultured with 0.8 mM Mg^{2+} (fig. 8Ba–c). The data provide direct evidence that magnesium deficiency activates TNF-α/NF-κB signaling in neurons.

Vincristine-induced Allodynia and Spinal Pathologic Synaptic Plasticity Are Prevented by Inhibition of NF-κB and Mimicked by Intrathecal Application of TNF-α

Having shown that vincristine injection activates TNF-α/NF-κB signaling in DRG and in the spinal dorsal horn and produces allodynia as well as CGRP-positive C-fiber sprouting, we next investigated their causal relationship, *i.e.*, if the nociceptive sensitization and the pathologic plasticity are the result of activation of TNF-α/NF-κB signaling. We found that intrathecal injection of NF-κB inhibitor (pyrrolidine dithiocarbamic acid, 10 ng in 10 μl) but not of vehicle 30 min before each vincristine injection substantially prevented the mechanical allodynia (fig. 9A). The injection of pyrrolidine dithiocarbamic acid also blocked vincristine-induced TNF-α up-regulation (fig. 9B) and CGRP-positive C-fiber from sprouting in the spinal dorsal horn (fig. 9Ca–f). The data suggest that vincristine-induced up-regulation of TNF-α and CGRP-positive C-fiber sprouting are dependent on the activation of NF-κB. Previous study shows that TNF-α is necessary and sufficient to induce LTP at C-fiber synapses in the spinal dorsal horn.[51,52] To test if TNF-α is also sufficient to induce CGRP-positive C-fiber sprouting and the region-dependent change in NR2B expression, rat recombination TNF-α (rrTNF-α, 3 μg in 10 μl) was intrathecally injected in naïve rats. We found that the injection of rrTNF-α but not of vehicle-induced mechanical allodynia and CGRP-positive

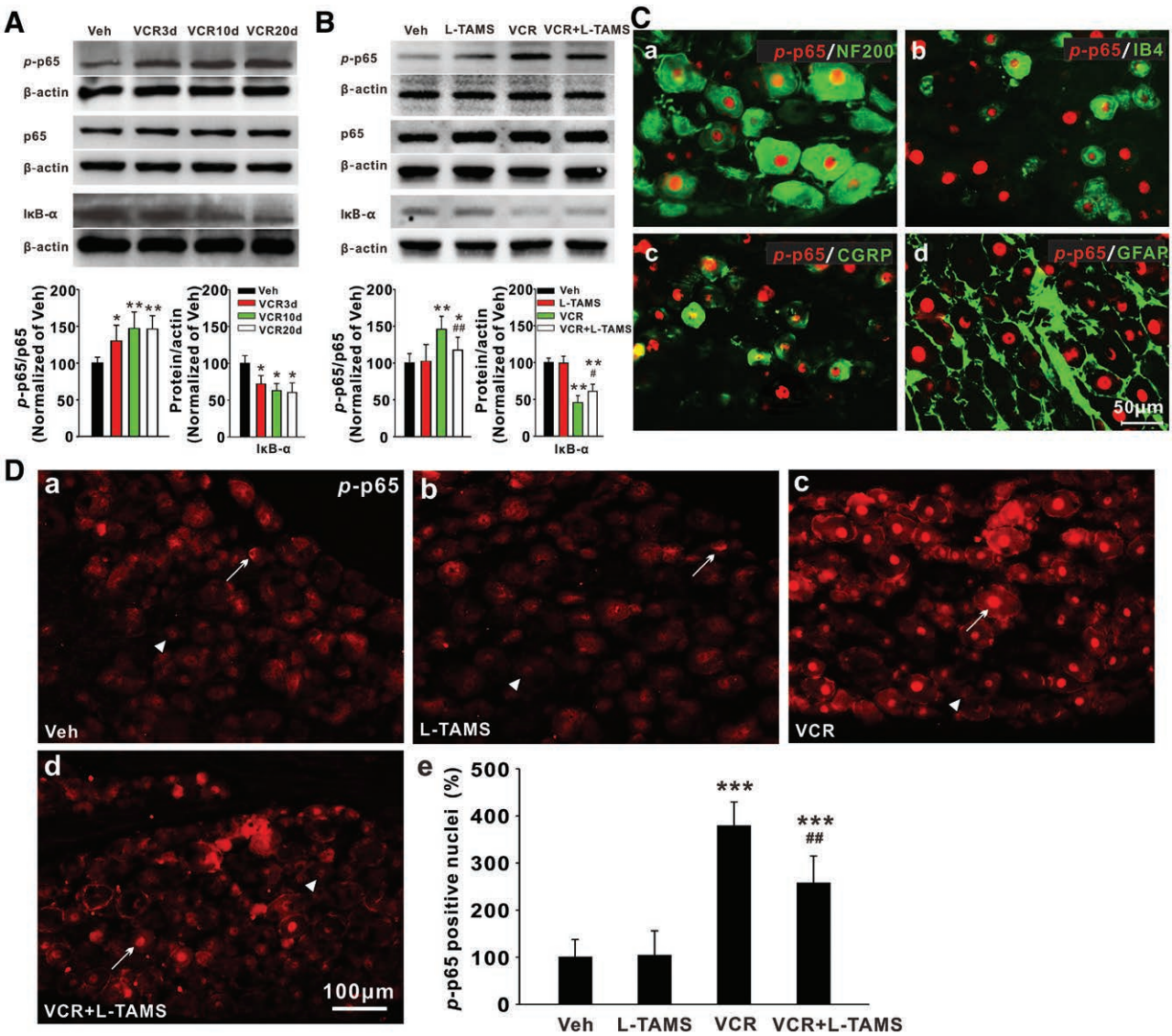

Fig. 6. Oral magnesium-L-threonate (L-TAMS) attenuates the activation of nuclear factor-κB (NF-κB) in dorsal root ganglion (DRG) neurons produced by vincristine (VCR). (*A*) The Western blots show the expression of NF-κB *p*-p65, total p65, and inhibitor of NF-κB (IκB-α) in DRG tissue at different time points after the onset of VCR injection. (*B*) The levels of *p*-p65, total p65, and IκB-α in DRGs of rats treated with vehicle (Veh), L-TAMS, VCR, or VCR + L-TAMS for 10 days are shown. (*C*) Double immunofluorescence staining shows that *p*-p65 was located in the nucleus of neuron (NF-200, IB4, and calcitonin gene–related peptide [CGRP]) but not of satellite glial cell (glial fibrillary acidic protein [GFAP]). (*D*) The representative photographs show the nuclear location of *p*-p65 in indicated groups (*a–d*). The *arrows* and *arrowheads* indicate the *p*-p65–positive and *p*-p65–negative nuclei that are defined by *p*-p65 integral optical density value being higher or lower than the mean value calculated in the Veh group (see Materials and Methods for details). The percentage changes of *p*-p65–positive nuclei among different groups are compared (*e*). n = 6 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the Veh group; #*P* < 0.05, ##*P* < 0.01, compared with the VCR group.

C-fiber sprouting in the dorsal horn, and the effects were substantially attenuated by oral application of L-TAMS (fig. 10A and Da–d). Furthermore, we found that TNF-α was up-regulated in both hippocampus and spinal dorsal horn tissues, while NR2B was up-regulated in spinal dorsal horn but down-regulated in hippocampus after intrathecal injection of rrTNF-α, and the changes were again blocked by L-TAMS (fig. 10, B and C). The data suggest that activation of TNF-α/NF-κB signaling in the spinal dorsal horn may contribute to the vincristine-induced nociceptive sensitization and pathologic plasticity in the dorsal horn.

Discussion

In the current study, we show for the first time that vincristine-induced mechanical allodynia and thermal hyperalgesia were accompanied by the reduction of extracellular and intracellular free Mg^{2+} (fig. 1) and the activation of

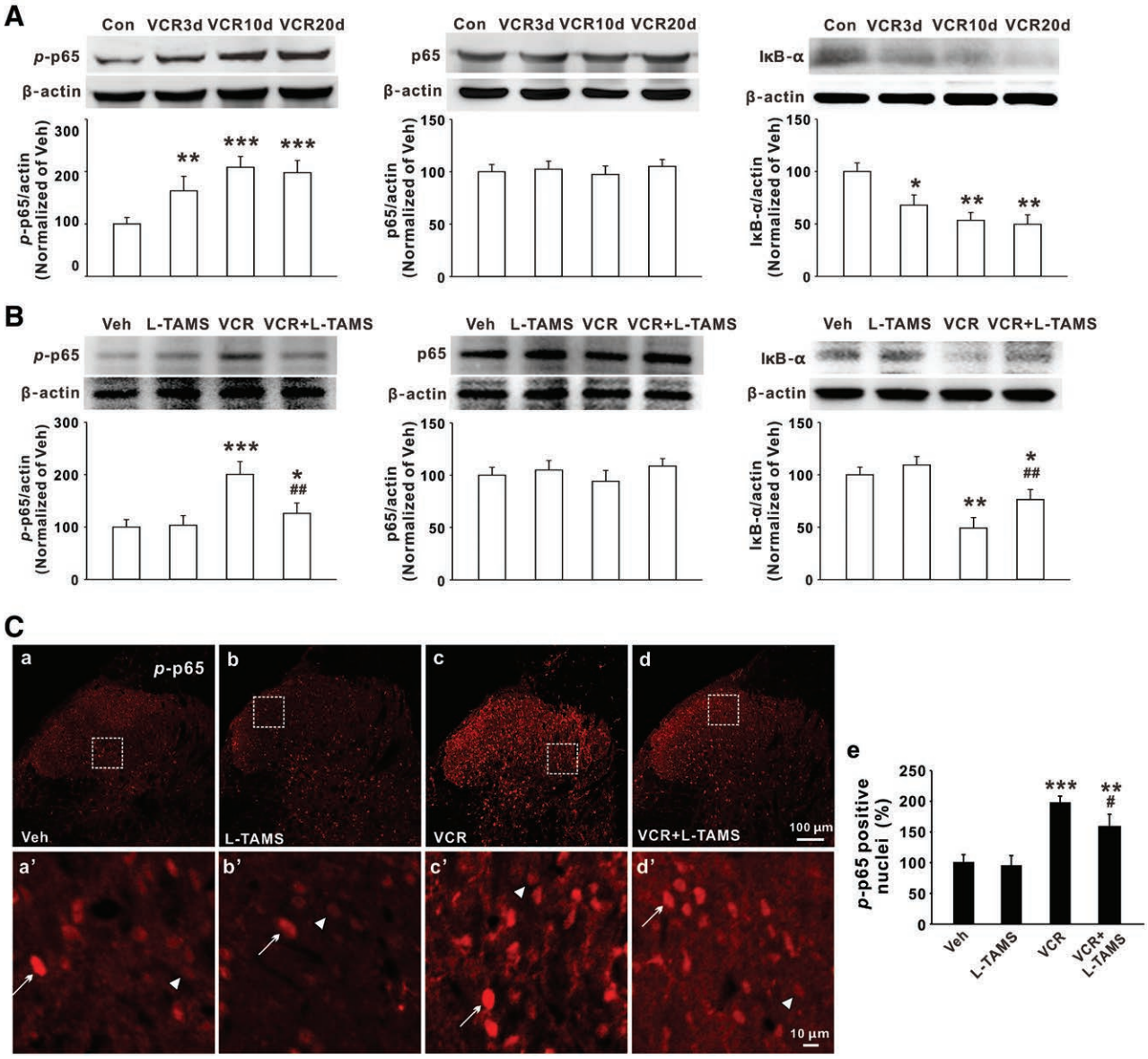

Fig. 7. Oral magnesium-L-threonate (L-TAMS) attenuates the activation of nuclear factor-κB (NF-κB) in dorsal horn neurons produced by vincristine (VCR). (*A*) The Western blots show the expression of NF-κB *p*-p65, total p65, and inhibitor of NF-κB (IκB-α) at different time points as indicated after the onset of VCR application. (*B*) The increased *p*-p65 and the decreased IκB-α induced by VCR were significantly attenuated but not reversed by L-TAMS. (*C*) The photographs show the nuclear location of *p*-p65 in different groups as indicated (*a–d*, *a′–d′*). The histogram (*e*) shows the changes in *p*-p65 nuclear translocation in different groups. The *arrows* and *arrowheads* indicate the *p*-p65–positive and *p*-p65–negative nuclei, respectively. The percentage changes of *p*-p65–positive nuclei among different groups are compared. n = 6 per group. ** $P < 0.01$, *** $P < 0.001$, compared with the vehicle (Veh) group; #$P < 0.05$, compared with the VCR group.

TNF-α/NF-κB in DRG and in the spinal dorsal horn (figs. 4–7). Reduction of Mg^{2+} activated TNF-α/NF-κB signaling in cultured DRG neurons (fig. 8). Chronic oral application of L-TAMS was able to prevent the magnesium deficiency and to attenuate the behavioral and molecular alterations produced by vincristine. Furthermore, we found that vincristine induced LTP at C-fiber synapses, the up-regulated NR2B subunit of NMDAR and CGRP-positive C-fiber sprouting in the spinal dorsal horn (figs. 2 and 3). The pathologic plastic changes were dependent on activation of TNF-α/NF-κB signaling and were also substantially prevented by oral application of L-TAMS. Taken together, vincristine reduces intracellular magnesium that induces nociceptive sensitization by activation of TNF-α/NF-κB signaling. Oral application of L-TAMS attenuates the vincristine-induced nociceptive sensitization by prevention of the magnesium deficiency. Vincristine activates TNF-α/NF-κB signaling by reduction of intracellular magnesium, leading to nociceptive sensitization, and the effects are inhibited by oral L-TAMS.

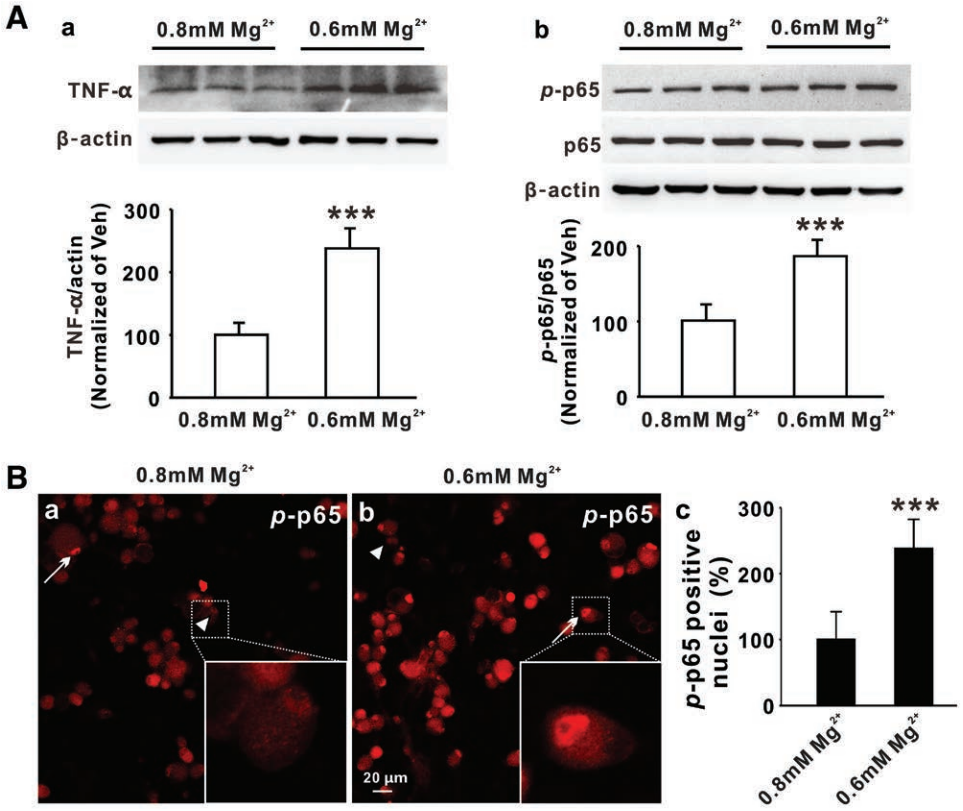

Fig. 8. Reduction of extracellular Mg^{2+} activates tumor necrosis factor-α (TNF-α)/nuclear factor-κB signaling in cultured dorsal root ganglion (DRG) neurons. (*A*) The Western blots show the expressions of TNF-α and *p*-p65 in the DRG neurons cultured with medium containing 0.6 and 0.8 mM Mg^{2+}, respectively. (*B*) The photographs show the nuclear location of *p*-p65 in DRG neurons cultured with 0.6 and 0.8 mM Mg^{2+} (*a, b*). The *arrows* and *arrowheads* indicate the *p*-p65–positive and *p*-p65–negative nuclei. The percentage changes of *p*-p65–positive nuclei among different groups are compared (*c*). n = 6 per group. ***$P < 0.001$, compared with the cultured with 0.8 mM Mg^{2+}.

As mentioned in the first paragraph, activation of TNF-α/NF-κB is critically involved in the nociceptive sensitization induced by nerve injury and paclitaxel. The current study shows that the activation of the pathway also contributes to the vincristine-induced nociceptive sensitization. However, the mechanisms, by which anticancer agents activate the TNF-α/NF-κB pathway, are poorly understood. In the current study, we provide evidence that vincristine treatment activated TNF-α/NF-κB signaling by reduction of intracellular free Mg^{2+} of DRG neurons. Because magnesium, as the second most abundant intracellular cation after potassium, is involved in more than 600 enzymatic reactions,[53,54] the mechanisms by which magnesium deficiency activates TNF-α/NF-κB signaling are complicated; dysfunction of many enzymes may be involved in the pathologic process. As magnesium deficiency is common in the patients receiving the anticancer agents, including cisplatin,[6] paclitaxel,[7] and Cetuximab,[8] oral application of L-TAMS may also be effective for prevention of neuropathic pain caused by these drugs. Further studies are needed to clarify these issues.

We found that oral application of L-TAMS completely blocked up-regulation of TNF-α but partially blocked activation of NF-κB induced by vincristine. This may explain why L-TAMS could attenuate but not completely prevent the vincristine-induced allodynia and hyperalgesia, as activation of NF-κB also leads to transcription of many other proinflammatory genes, such as interleukin-1β, which are also important for nociceptive sensitization.[24]

It is worth noting that TNF-α/NF-κB signaling is critical for many physiologic functions, such as immunity, cell proliferation, differentiation, and apoptosis.[49] Accordingly, the stratagem to treat the related diseases should normalize but not simply inhibit the pathway. This is difficult to achieve with NF-κB inhibitors. L-TAMS exerts its effect by prevention of magnesium deficiency that activates TNF-α/NF-κB signaling and therefore is able to block the overproduction of TNF-α in hippocampus induced by peripheral nerve injury[16] and the activation of TNF-α/NF-κB signaling produced by vincristine treatment in DRG and the spinal dorsal horn (current study) but does not affect the pathway in physiologic conditions. In addition, clinical studies show that supplementation of Mg^{2+} does not reduce the anticancer efficiency of chemotherapeutic agents.[9,10] In the current study, no obvious side effect, such as diarrhea, which is commonly induced by other magnesium compounds, was

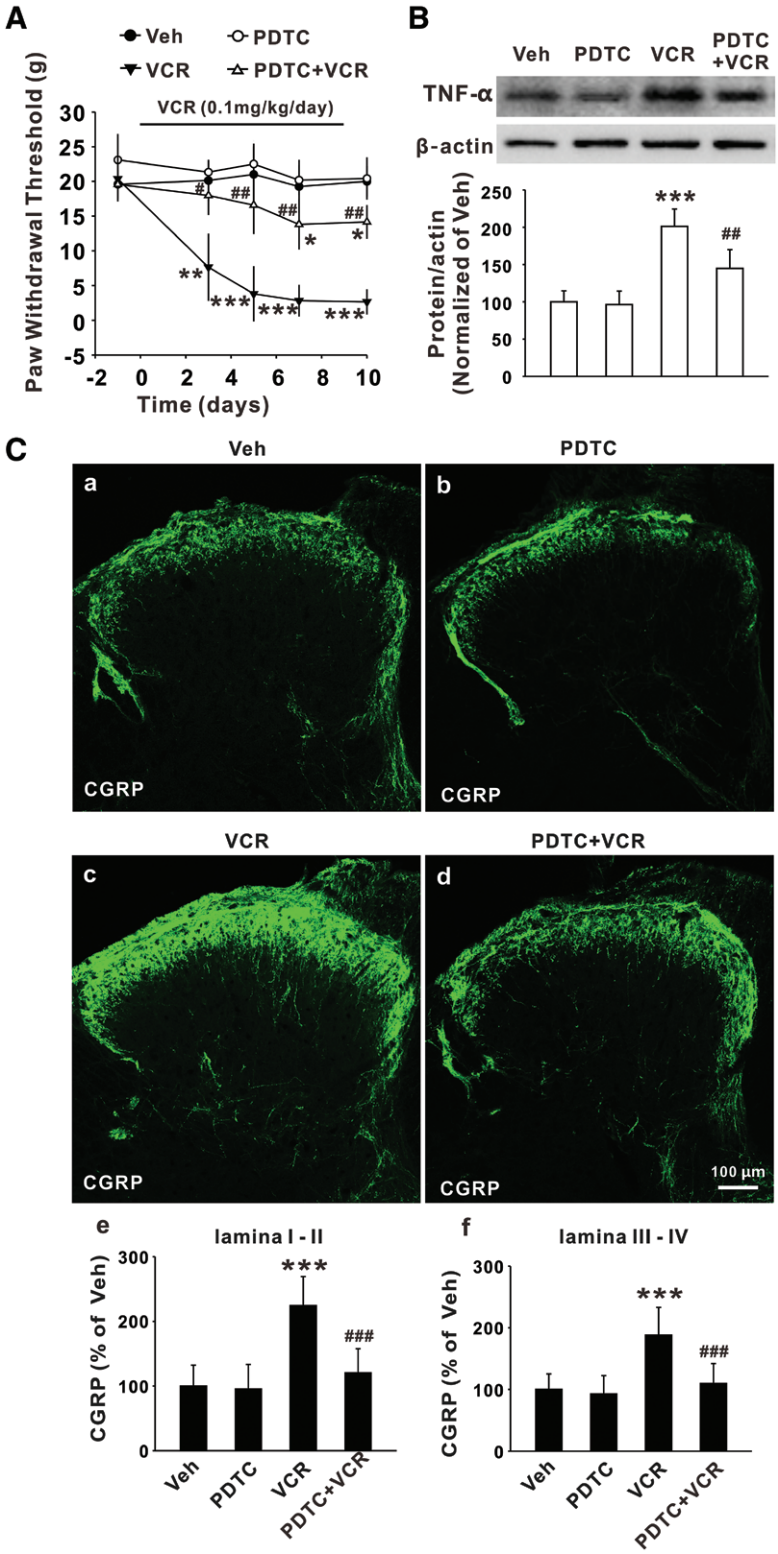

Fig. 9. Vincristine (VCR)-induced allodynia, up-regulation of tumor necrosis factor-α (TNF-α), and calcitonin gene–related peptide (CGRP) C-fiber sprouting in the dorsal horn are abolished by intrathecal injection of nuclear factor-κB (NF-κB) inhibitor. (*A*) The time courses of paw withdrawal thresholds in vehicle (Veh), pyrrolidine dithiocarbamic acid (PDTC), VCR, and PDTC + VCR groups are shown. (*B*) The Western blots show the levels of TNF-α in different groups, and the histograms show the summary data. (*C*) The photographs show CGRP C-fibers in the dorsal horn of different groups (*a–d*), and the histograms show that the up-regulation of CGRP in both laminas I to II and laminas III to IV induced by VCR was blocked by NF-κB inhibitor PDTC (*e–f*). n = 6 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the Veh group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, compared with the VCR group.

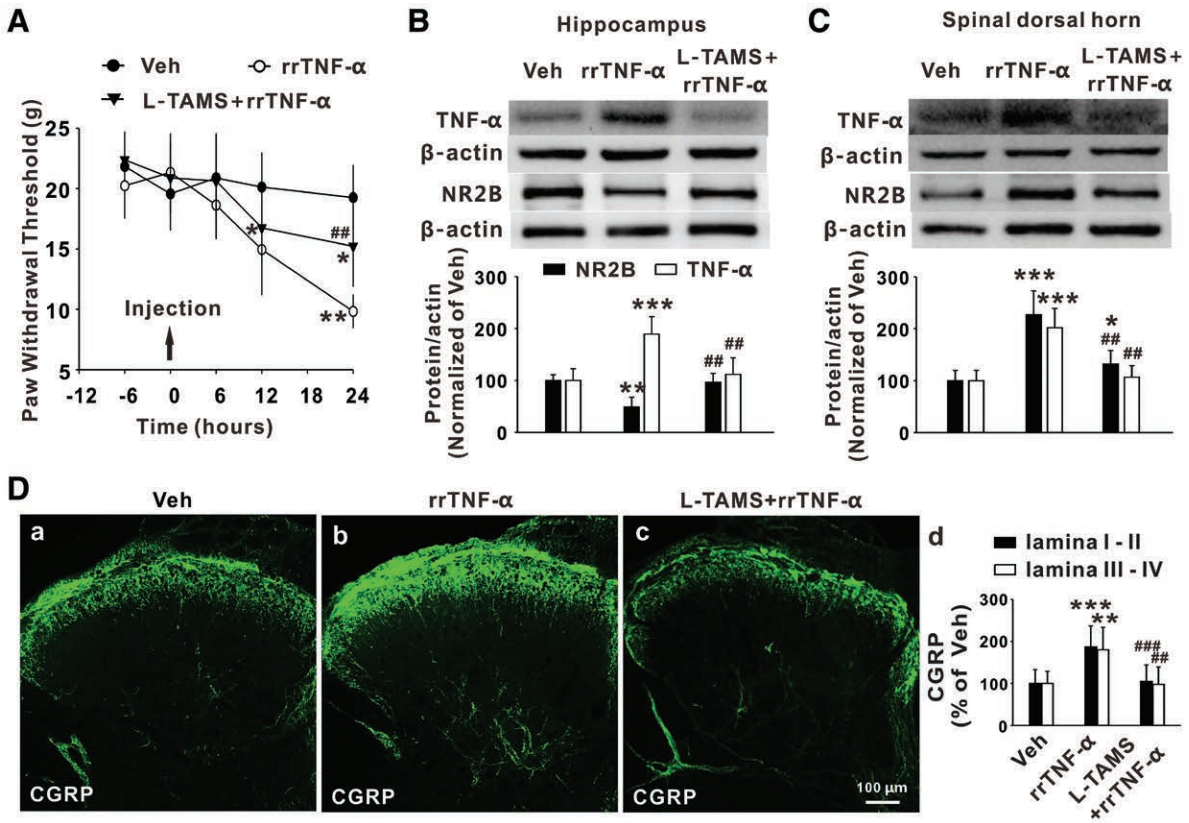

Fig. 10. Intrathecal injection of rat recombination tumor necrosis factor-α (rrTNF-α) induces mechanical allodynia and spinal pathologic synaptic plasticity, and the effects are attenuated by oral magnesium-L-threonate (L-TMAS). (*A*) The time courses of paw withdrawal thresholds in vehicle (Veh), rrTNF-α, and L-TAMS + rrTNF-α groups are shown (n = 5 per group). (*B*, *C*) The protein levels of TNF-α and *N*-methyl-D-aspartate receptor type 2B (NR2B) in different groups are shown (n = 5 per group). (*D*) Intrathecal injection of rrTNF-α induced calcitonin gene–related peptide (CGRP) C-fiber sprouting, the effect was blocked by oral L-TAMS (n = 5 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the Veh group; ##*P* < 0.01, ###*P* < 0.001, compared with the rrTNF group.

observed. Therefore, oral application of L-TAMS is a safe and effective means to prevent painful CIPN.

LTP at C-fiber Synapses and Peptidergic C-fiber Sprouting in the Dorsal Horn May Contribute to the Nociceptive Sensitization Induced by Vincristine

The clinical manifestation of CIPN is severe pain accompanied by the loss of tactile sensation in the distal extremities.[55,56] The mechanisms underlying the opposite change in pain and tactile sensation are not fully understood. Many anticancer drugs, including vincristine and paclitaxel, inhibit axonal transport mediated by microtubules. Vincristine induces a significant decrease in axonal microtubules in myelinated fibers[47] and reduces the number of afferent myelinated fibers in the sural nerve biopsy from human patients[57] and in the sciatic nerve of mice.[58] Therefore, damage of myelinated fibers may contribute to the loss of tactile sensation. However, why the tactile sensation loss is accompanied by severe pain is poorly understood. It has been shown that vincristine does not affect axonal microtubules in unmyelinated C-fibers.[48] In contrast, paclitaxel[59] and vincristine (current study) induce spinal LTP at C-fiber synapses. Importantly, we found that CGRP-positive C-fibers in the spinal dorsal horn were substantially increased and sprouted into the deep lamina in vincristine-treated rats. It is well established that the CGRP-positive nociceptor, expressing TrkA (a receptor of nerve growth factor) and substance P, makes direct synaptic contact with the neurokinin 1 receptor–positive neurons in the dorsal horn.[44] The neurokinin 1–positive neurons that project to the thalamus and neocortex are proved essential for development of nociceptive sensitization induced by inflammation and nerve injury.[60,61] Therefore, we suggest that CGRP-positive fiber sprouting is the structural basis of the long-lasting LTP at C-fiber synapses that leads to the behavioral nociceptive hypersensitivity produced by chemotherapy.

The mechanisms underlying the peptidergic C-fiber sprouting is largely unknown at present. As we showed that TNF-α/NF-κB signaling was activated in DRG and in the spinal dorsal horn after vincristine injection and that the CGRP C-fiber sprouting was substantially blocked by NF-κB inhibitor and mimicked by rrTNF-α, we suggest that activation of the TNF-α/NF-κB pathway may be responsible for the pathologic plasticity. The TNF-α/NF-κB

pathway has long been considered as a prototypical proinflammatory signaling pathway. Activation of this pathway leads to up-regulation of many proinflammatory factors including cytokines, chemokines, and adhesion molecules,[62] which may create and maintain an inflammatory microenvironment in the spinal dorsal horn. The microenvironment may lead to the peptidergic C-fiber sprouting observed in the current study. Like nociceptive sensitization, the vincristine-induced C-fiber sprouting was again substantially attenuated but not abolished by chronic oral application of L-TAMS.

L-TAMS May Attenuate Vincristine-induced Spinal LTP and Nociceptive Sensitization by Prevention of NR2B Up-regulation in the Spinal Dorsal Horn

Activation of postsynaptic NMDAR is important for formation of memory and nociceptive hypersensitivity. As the NMDAR channel can be blocked by Mg^{2+},[63] increasing Mg^{2+} would attenuate both of them. However, our previous work shows that elevating brain Mg^{2+} by oral application of L-TAMS improves memory function and enhances NMDAR activity and NR2B (but not NR2A of NMDAR) expression in the hippocampus of naive rats.[14] Oral L-TAMS also prevents and restores the deficits of short-term memory and of LTP in the hippocampus induced by peripheral nerve injury.[16] Enhancing NMDAR function by up-regulation of NR2B expression in the hippocampus is believed to contribute to a beneficial effect of L-TAMS on memory. A previous study shows that up-regulation of NR2B (but not NR2A) in the spinal dorsal horn is critical for development of hyperalgesia.[43] If L-TAMS also up-regulates NR2B expression in the spinal dorsal horn, it would induce but not inhibit nociceptive synaptic transmission. In contrast to this assumption, the current study shows that oral L-TAMS attenuated the allodynia and hyperalgesia induced by vincristine but did not attenuate the nociceptive behaviors in the vehicle-treated rats. To explore the reason for this discrepancy, we measured the NR2B expression in the hippocampus and in the spinal dorsal horn and found that NR2B expression was down-regulated in the hippocampus but up-regulated in the spinal dorsal horn 10 days after vincristine injection, and the opposite changes were prevented by oral L-TAMS, while the oral L-TAMS did not affect NR2B expression in rats receiving saline injection. The data demonstrate that oral L-TAMS prevents abnormal expression of NR2B but does not affect its basal level in both the hippocampus and the spinal dorsal horn. The pharmacologic property of L-TAMS may count for its inhibitory effect on nociceptive sensitization and predicts that it may also prevent vincristine-induced memory deficits.[64]

How could vincristine oppositely affect NR2B expression in hippocampus and in spinal dorsal horn? Our previous study shows that TNF-α is up-regulated in the hippocampus of the spared nerve injury model of neuropathic pain.[16,65] The current study reveals that vincristine injection up-regulated TNF-α in DRGs and the spinal dorsal horn. Importantly, we have shown that intrathecal injection of rrTNF up-regulated TNF-α in both the hippocampus and the spinal dorsal horn but down-regulated NR2B in the hippocampus and up-regulated NR2B in the spinal dorsal horn. Accordingly, overproduction of TNF-α may be responsible for the vincristine-induced opposite changes in NR2B expression. Although the mechanisms, by which TNF-α oppositely regulates NR2B in the hippocampus and in the spinal dorsal horn, remain elusive, we provide evidence that oral application of L-TAMS prevents vincristine-induced NR2B up-regulation in the spinal dorsal horn, which may contribute to its inhibitory effect on allodynia and hyperalgesia. Inhibition of TNF-α overproduction may contribute to the effects. We cannot exclude the possibility that elevating Mg^{2+} may inhibit nociceptive sensitization by direct blockade of postsynaptic NMDAR.

In conclusion, vincristine induced mechanical allodynia and thermal hyperalgesia by activation of the TNF-α/NF-κB pathway *via* reducing extracellular and intracellular free Mg^{2+} in nervous system. Chronic oral application of L-TAMS attenuated the vincristine-induced nociceptive sensitization by prevention of the magnesium deficiency.

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Competing Interests

Dr. G. Liu declares that he is a co-founder of NEUROCENTRIA, Fremont, California, a company whose goal is to develop drugs to treat age-dependent memory decline and Alzheimer disease. He also reports his United States patent application on magnesium-L-threonate. The other authors declare no competing interests.

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Address correspondence to Dr. X.-G. Liu: Department of Physiology and Pain Research Center, Zhongshan School of Medicine, Sun Yet-Sen University, Guangzhou, 510080, China. liuxg@mail.sysu.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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