CaMKII and Cav3.2 Mediate Cx43-Dependent Inflammation by Activating Astrocytes in Vincristine-Induced Neuropathic Pain

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Abstract

Vincristine (VCR), an alkaloid isolated from vinca, is a commonly used chemotherapeutic drug. However, treatment with VCR can lead to dose-dependent peripheral neurotoxicity, mainly manifesting as neuropathic pain, which is one of the dominant reasons for limiting its utility. Experimentally, we discovered that VCR-induced neuropathic pain was accompanied by astrocyte activation; the upregulation of p-CaMKII, Cav3.2, and Cx43 expression; and the production and release of inflammatory cytokines and chemokines in the spinal cord. Similar situations were also observed in astrocyte cultures. Interestingly, these alterations were all reversed by intrathecal injection of KN-93 (a CaMKII inhibitor) or L-Ascorbic acid (a Cav3.2 antagonist). In addition, KN-93 and L-Ascorbic acid inhibited the increase in \([\text{Ca}^{2+}]_i\) associated with astrocyte activation. We also verified that inhibiting and knocking down Cx43 levels via intrathecal injection of Gap27 and Cx43 siRNA relieved pain hypersensitivity and reduced the release of inflammatory factors; however, they did not affect astrocyte activation or p-CaMKII and Cav3.2 expression. The overexpression of Cx43 through the transfection of the Cx43 plasmid did not affect p-CaMKII or Cav3.2 expression in vitro. Therefore, CaMKII and Cav3.2 may activate astrocytes by increasing \([\text{Ca}^{2+}]_i\), thereby mediating Cx43-dependent inflammation in VCR-induced neuropathic pain. Moreover, we demonstrated that the CaMKII signalling pathway was involved in VCR-induced inflammation, apoptosis, and mitochondrial damage. In summary, our findings show a novel mechanism by which CaMKII and Cav3.2 mediate Cx43-dependent inflammation by activating astrocytes in neuropathic pain induced by VCR.

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a significant health concern that poses severe challenges to cancer patients' physical and mental health and represents a colossal burden to society and the economy worldwide (Sisignano et al. 2014, Cavaletti et al. 2019). Vincristine (VCR), an alkaloid isolated from vinca, has long been applied to treat various cancers, such as neuroblastomas, lymphomas, and leukaemias (Kantarjian et al. 2004, Shimizu et al. 2017). As a commonly used chemotherapeutic drug, the pharmacological mechanism of VCR is to disrupt microtubule formation in mitotic spindles, leading to the cessation of cellular mitosis (Gupta et al. 2006). Furthermore, VCR plays an anticancer role by inducing mitochondrial dysfunction and subsequent energy deficiency (Xiao and Bennett 2012). However, treatment with VCR can lead to dose-dependent peripheral neurotoxicity, mainly manifesting as somatosensory and motor dysfunction, which is one of the dominant reasons for limiting its utility (Schouten et al. 2020, Balayssac et al. 2011). At present, due to an insufficient understanding of the pathogenesis of neuropathic pain caused by VCR, there is a lack of effective prevention and treatment strategies. Existing studies have shown that VCR causes pathological changes in the mouse spinal cord, such as glial cell activation, intracellular ion disorders, inflammation, and apoptosis (Gold and Gebhart 2010, Zhang et al. 2013, Surmeier, Guzman and Sanchez-Padilla 2010, Chen, Wang and Song 2020).
Neuropathic pain is generally considered a manifestation of the neuroplasticity of the primary sensory neurons of the central nervous system (CNS) (Gold and Gebhart 2010). However, in recent decades, increasing research has confirmed glial cells’ significance in maintaining neuropathic pain by modulating synaptic transmission (Nedergaard and Verkhratsky 2012). In the CNS, astrocytes are the most abundant type of glial cells. Many papers have certified that they are involved in developing and maintaining neuropathic pain by releasing astroglial mediators to upregulate nociceptive neuron activity in the spinal cord (Gao, Ji and therapeutics 2010). Under spinal cord injury or nerve injury, astrocytes exhibit persistent reactive changes and promote neuropathic pain by producing proteases, inflammatory cytokines, and chemokines (Kawasaki et al. 2008, Guo et al. 2007, Zhang et al. 2013).

The activation of astrocytes requires the participation of various ions, including calcium ions (Ca\(^{2+}\)) that regulate a series of physiological responses of neurons via neurotrophic factors and neurotransmitters (Surmeier et al. 2010, Neher and Sakaba 2008). Intracellular Ca\(^{2+}\) homeostasis imbalance leads to impaired plasticity, synaptic dysfunction, and neuronal degeneration (Nedergaard, Rodríguez and Verkhratsky 2010, Marambaud, Dreses-Werringloer and Vingtdeux 2009). Calcium/calmodulin-dependent protein kinase II (CaMKII) is a widely expressed multifunctional serine/threonine kinase that regulates the transcription of Ca\(^{2+}\) channels (Naranjo and Mellström 2012). Ca\(^{2+}\)-dependent CaMKII modulates the activity or plasticity of neurons by Ca\(^{2+}\) signalling in the development of neuropathic allodynia (Song et al. 2010, Kim and Sharma 2004). Voltage-dependent calcium channel 3.2 subunit (Cav3.2), a low-threshold calcium channel, is the molecular substrate of neuropathic allodynia in lamina II and III of the spinal dorsal horn (François et al. 2015) and a key regulator of Dorsal root ganglion (DRG) neuronal excitability (McCallum et al. 2003). Cav3.2 antisense oligonucleotide injected into the sheath to reduce the t-type calcium current of DRG neurons was shown to relieve the traumatic response of neuropathic pain in rats (Bourinet et al. 2005). Therefore, we hypothesized that CaMKII and Cav3.2 are involved in VCR-induced neuropathic pain by activating astrocytes by regulating intracellular free calcium ([Ca\(^{2+}\)]\(_i\)).

Chemotherapy agents have been shown to induce spinal cord neuroinflammation and apoptosis in mice (Chen et al. 2020, Li et al. 2020). Recent studies suggested that decreasing the phosphorylation of CaMKII could inhibit NF-κB signalling pathway activation and diminish lipopolysaccharide-induced neuroinflammation in primary microglial cells (Park, Jang and Park 2020). Furthermore, inhibiting the expression of CaMKIIβ improved cell viability and reduced the apoptosis rate, which protected DRG cells from ropivacaine hydrochloride-induced neurotoxicity injury (Wen et al. 2019). The interaction between reactive oxygen species (ROS) and cellular Ca\(^{2+}\) activates related signalling pathways, leading to neuronal apoptosis (Cheng et al. 2012b, Franklin 2011). However, whether VCR causes inflammation and apoptosis through the CaMKII signalling pathway needs further verification.

Astrocytes form interconnected networks coupled in the adult CNS through connexin 43 (Cx43), which is a major structural component of gap junctions (Bennett et al. 2012). There is now evidence that gap junctions are involved in chronic pain induced by tissue inflammation or nervous system damage (Wu et al. 2012). Our previous research verified that VCR upregulated the expression of Cx43 (Zhou et al. 2020).
However, it is still unclear how Cx43 participates in and maintains neuropathic pain induced by VCR. Therefore, in this experiment, we intended to investigate the roles and mechanisms of CaMKII, Cav3.2, and Cx43 in VCR-induced neuropathic pain.

2. Materials And Methods

2.1. Animals

Adult male ICR mice weighing 20 to 25 g were provided by Qinglongshan Animal Farm (Nanjing, China). Mice were housed under a fixed 12-h/12-h on/off light cycle with controlled room temperature and relative humidity (22–25 °C, 60 ± 10%) and allowed free access to a standard diet and water. All animal experimental procedures in this study were performed following the ARRIVE guidelines and complied with the policies of the International Association for the Study of Pain. All experiments complied with the Guidelines for the Care and Use of Laboratory Animals and were approved by China Pharmaceutical University (Nanjing, China; licence number: SYXK (Su) 2016-0011).

2.2. Establishment of the VCR-induced pain model and drug administration protocols

Mice were intraperitoneally injected with VCR sulfate (0.1 mg/kg; Shenzhen Main Luck Pharmaceutical Incorporated, China; dissolved in normal saline) once a day for five consecutive days (Shen et al. 2015).

KN-93 (a CaMKII inhibitor, 70 nM, 10 µl; Selleck, Houston, USA; intrathecal), Gap27 (a synthetic Cx43 mimetic peptide; 14.4, 43.2, and 144 nM, 10 µl each; MedChemExpresswere, New Jersey, USA) and L-Ascorbic acid (a Cav3.2 channels inhibitor; 30, 100, and 300 µM, 10 µl each; Tocris Bioscience,, Bristol, UK; intrathecal) were administered into the dorsal subarachnoid space of animals daily for 2 consecutive days from day 6 after the first-day vincristine injection. The control groups were injected with intrathecal dimethyl sulfoxide or saline.

Cx43-specific small interfering RNA (siRNA) was provided by RiboBio (Guangzhou, China). Cx43 siRNA (50 nM, 10 µl) was dissolved in the transfection reagent riboFECT™ CP and intrathecally injected daily for 2 consecutive days from day 6 after the first-day VCR injection. Missense siRNA was administered intrathecally in the same way as a sham treatment.

2.3. Behavioural assessments

In this experiment, we mainly evaluated the behaviour of mice with mechanical allodynia and heat hyperalgesia in the same quiet behavioural testing room. For the mechanical allodynia assessment, an ascending series of von Frey filaments (0.07-2.0 g, Woodland Hills, Los Angeles, CA) was used to determine the mechanical withdrawal threshold for all mice (Dixon 1980). Mice were placed in transparent plexiglass compartments on an elevated metal grid and acclimated for 30 min before testing. In the mice's peaceful state, each monofilament was vertically stimulated 5 times in ascending order to a plantar surface (2–3 s for each filament). Dixon's up-down method was applied to determine the 50%
paw withdrawal threshold (PWT) when the monofilament stimulation led to paw licking or withdrawal in 3 of 5 applications. Heat hyperalgesia was assessed as infrared stimulation duration and measured using the PL-200 stinging instrument (TechMan, Chengdu, China). The intensity of the radiant heat was adjusted to ensure that the paw withdrawal latency (PWL) of the sham group fell in the range of 10 ± 2 s according to a previous research (Hu et al. 2017).

Behavioural evaluations were performed from 0 to 21 days after the first intraperitoneal injection of VCR. For the groups treated with KN-93, Gap27, L-Ascorbic acid, and Cx43 siRNA, behaviour was assessed at 2 h after the intrathecal injections.

2.4. Primary astrocyte cultures

The method of primary astrocyte culture was previously used and described. Briefly, the cerebral cortex of neonatal mice (within 48 h) was harvested under sterile conditions. The cortical hemispheres were cut into pieces after careful removal of the meninges and then digested with papain (2 mg/ml) for 30 minutes, filtered by centrifugation, and resuspended in DMEM/F-12 medium (Wisent, Canada) containing 10% (v/v) foetal bovine serum (FBS) (AusGeneX, Molendinar, Australia). Cells were maintained at 37 °C with 5% CO

2.5. Immunofluorescence Staining

Mice were anaesthetized with isoflurane and transcardially perfused through the ascending aorta with PBS containing 1.5% picric acid and 4% paraformaldehyde. Then, a part of the lumbar spinal cord of the mouse was removed and post fixed with the same fixative for 24 h and then dehydrated with 30% sucrose solution. Spinal cord slices (25 µm) were prepared using a cryostat (Thermo Fisher Scientific, Waltham, USA) and stored in 50% glycerol at -20 °C until immunofluorescence staining. In brief, the sections were blocked/permeabilized with PBS (pH 7.4) containing 5% normal goat serum and 0.3% Triton X-100 for 30 min at room temperature and then incubated overnight at 4 °C with the following primary antibodies: p-CaMKII antibody (1:100, rabbit; rabbit; Abcam, Cambridge, UK), Cav3.2 antibody (1:50, rabbit; Thermo Fisher Scientific, Waltham, USA) and glial fibrillary acidic protein (GFAP) antibody (1:300, mouse; Cell Signalling Technology, Danvers, USA). Then, the sections were washed three times with PBS and incubated with Cy3- (cyanine 3) or FITC-conjugated secondary antibodies (1:100; Bioss, Beijing, China) for 2 h at room temperature in the dark. For double immunofluorescence staining, sections were incubated with a mixture of primary antibodies, followed by a mixture of Cy3- and FITC-conjugated secondary antibodies. Finally, the stained sections were visualized using a Carl Zeiss LSM 5 PASCAL.
laser scanning confocal microscope. Data were processed using ImageJ 1.50i software (National Institutes of Health, USA) at the appropriate intensity to display all positive cells.

For the immunofluorescence staining of cultured astrocytes, astrocytes were fixed with 4% paraformaldehyde for 15 min and processed for immunofluorescence staining with GFAP antibody (1:200), as indicated above.

2.6. Western blotting

Twenty milligrams of frozen spinal segment tissue was ground in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotech, Nantong, China) containing proteinase and phosphatase inhibitors (Applygen Technologies, Beijing, China). Cells were seeded onto six-well plates, and 100 µl RIPA lysis buffer containing proteinase inhibitors was added to each well. The samples were centrifuged at 12000 rpm for 15 min after 30 minutes of lysis on ice. The protein concentration was measured by a BCA Protein Assay Kit (Beyotime Biotech, Nantong, China). Each lane was loaded with the same amount of protein (50 g), which was separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes. After transfer, 5% skimmed milk (BD Biosciences, San Diego, CA) was used to saturate the blots for 60 min. Then, the blots were incubated overnight at 4 °C with primary antibodies against NF-κB p65, phospho-NF-κB p65, Bax, Bcl-2 cleaved caspase-3, Cx43 (1:1000, rabbit; Cell Signalling Technology, Danvers, USA), Cav3.2 (1:500, rabbit; Thermo Fisher Scientific, Waltham, USA), CaMKII, phospho-CaMKII (1:1000, rabbit; Abcam, Cambridge, UK), and GAPDH (1:10000, rabbit; Proteintech Group, Wuhan, China), followed by the addition of horseradish peroxidase-conjugated secondary antibody (1:2500) for 2 h. The blots were visualized using an enhanced chemiluminescence system (Bio-Rad, Hercules, CA, USA). The band intensities were measured to perform densitometric analysis using ImageJ 1.51 software (Rawak Software Inc., Stuttgart, Germany).

2.7. Quantitative Reverse Transcription-Polymerase Chain Reaction

According to the reagent instructions, total RNA of spinal cord tissues and cells was isolated using TRIzol (TaKaRa, Tokyo, Japan). PrimeScript™ Reverse Transcriptase (TaKaRa, Tokyo, Japan) was used to generate complementary DNA (cDNA). Subsequently, real-time quantitative PCR was performed using a QuantStudio 3 Real-time PCR System (Applied Biosystems, Foster, CA, USA) with SYBR Green Master Mix (Vazyme, Nanjing, China). In this study, the primer sequences are listed in Table 1. PCR amplifications were performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Levels of each sample mRNA were calculated using the delta method from threshold cycle numbers after mRNA normalization to GAPDH. The relative messenger RNA (mRNA) levels were calculated according to the $2^{-\Delta\Delta CT}$ process.
| Gene          | Primer Sequence (5’-3’) |
|--------------|------------------------|
| Mouse TNF-α  | F: TCCCCAAAGGGATGAGAAG |
|              | R: CACTTGGTGGTTTGCTACGA |
| Mouse IL-6   | F: ACAAAGCCAGAGTCTCTGAGGAG |
|              | R: TTGGATGGTCTTTTGGTCTAGCA |
| Mouse IL-1β  | F: ACTGTGAAATGCCACCTTTTG |
|              | R: TGTTGATGTGCTGCTGCTGAG |
| Mouse CXCL12β| F: TGCATCAGTGAGGTAAACCA |
|              | R: TTCTTCAGCCGTGCAACAATC |
| Mouse Bax    | F: TGGAGATGAACTGGACAGATAT |
|              | R: GCAAAATGAAGGGGCAACCAC |
| Mouse Bcl-2  | F: CTCAGGCTGGAAGGAGGAGAT |
|              | R: AAGCTGTCACAGGAGGGCTAC |
| Mouse Cx43   | F: GGTGATGAACAGTCTGCCTTCG |
|              | R: GTGAGCCAAATGAGGAGGTGG |
| Mouse Cav3.2 | F: GTTCGTGCTGTCATGAGGAGGT |
|              | R: GGCTTTCTGCTGTAGGTAGGT |
| Mouse GAPDH  | F: AGGTTCGCGTGGAAACGGATTG |
|              | R: TGTAGACCATGTAGGTGAGGTCA |

### 2.8. Enzyme-linked Immunosorbent Assay Kits

Enzyme-linked immunosorbent assay (ELISA) kits (Dakewe Biotech, Beijing, China) were used to analyse IL-6 and IL-1β levels in serum and cell culture media according to the instructions provided by the manufacturers.

### 2.9. Cell Counting Kit-8 Assay

Cell viability was analysed by a Cell Counting Kit-8 Assay (CCK-8) analysis kit (KeyGen Biotechnology, China). Briefly, primary astrocytes were treated with VCR at different concentrations (0.1 nM to 100 nM) for 24 h, and then 10 µl CCK-8 reagent was added to the medium and incubated for 2 h. After that, the absorbance was detected at 450 nm with a Multiskan FC microplate reader (Thermo Fisher, Waltham, MA, United States).
2.10. Measurement of intracellular free calcium ([Ca^{2+}]_i) level

[Ca^{2+}]_i was determined with the calcium-sensitive dye Fluo-4/AM (Beyotime, Shanghai, China). After treatment, the astrocyte cells were washed thrice with cold sterile HBSS, loaded with 5 µM Fluo-4/AM and incubated at 37 °C for 45 min. The cells were then washed twice with HEPES buffer solution containing (mM) 10 HEPES, 5 KCl, 137 NaCl, 0.5 MgCl_2, 1 CaCl_2, 1 Na_2HPO_4, 1% BSA, and 10 glucose, pH 7.4. Finally, the cells were incubated in HBSS with 1% serum for 40 min to complete dye de-esterification, followed by detection under a SynergyH1 microplate reader (BioTek, Vermont, USA).

2.11. TUNEL assay

A TUNEL in situ cell death detection kit (Roche, USA) was used to calculate the cell apoptosis rate in situ according to the protocols recommended by the manufacturer. TUNEL-positive signals showing green fluorescence were detected by laser scanning confocal microscopy.

2.12. Annexin V/PI double staining

After treatment, astrocytes were rinsed three times with cold PBS and then double-stained using an Annexin V-FITC Apoptosis Detection Kit (San Diego, CA) according to the manufacturer's protocols. After incubation at room temperature for 15 minutes in the dark, apoptotic cells were detected using a flow cytometer (BD Biosciences, San Diego, CA).

2.13. Mitochondrial membrane potential (MMP) determination

The MMP △Ψm of cells was examined by a mitochondrial membrane potential assay kit with JC-1 (Beyotime, Shanghai, China), followed by imaging under flow cytometry.

2.14. Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Differences between groups were compared using unpaired two-tailed Student’s *t*-test or one-way analysis of variance (ANOVA). SPSS (version 22.0) and GraphPad Prism software (version 8.0.1) were used to analyse the data and graphing, respectively. A value of *p* < 0.05 was deemed statistically significant.

3. Result

3.1 VCR treatment induces persistent pain hypersensitivity, astrocyte activation, inflammation and apoptotic.
The in vivo experimental process is exhibited in Fig. 1A. Mice were intraperitoneally injected with VCR daily for 5 consecutive days, and pain behaviour was monitored over time. At three days after starting intraperitoneal administration of VCR, the results revealed evident pain hypersensitivity ($p < 0.05$), which was characterized by mechanical allodynia and heat hyperalgesia (Fig. 1B, C). For mechanical sensitivity, compared with mice treated with saline (sham group), the paw withdrawal threshold in response to von Frey hair stimulation in VCR-treated mice (VCR group) was fully decreased at day 7 ($1.43 \pm 0.08$ g vs. $0.35 \pm 0.07$ g; $p < 0.05$) and persisted at 21 days ($1.44 \pm 0.11$ g vs. $0.84 \pm 0.13$ g; $p < 0.05$) (Fig. 1B). For heat sensitivity, the paw withdrawal latency in response to the heat stimulation in the VCR group was reduced wholly compared with that in the sham group at day 7 ($11.5 \pm 0.30$ s vs. $7.3 \pm 0.32$ s; $p < 0.05$) and day 21 ($12.8 \pm 0.51$ s vs. $11.13 \pm 0.31$ s; $p < 0.05$) after the first administration of VCR (Fig. 1C).

On days 3, 7, 14, and 21, we analysed astrocyte activation and the inflammatory response. Astrocyte reactivity to VCR was evaluated via GFAP expression in the spinal cord. Compared with resting-state GFAP-positive astrocytes in the sham group, numerous GFAP-positive astrocytes in the VCR group showed intense immunoreactivity, and the increase in GFAP-positive astrocytes was most pronounced at seven days ($6.56 \pm 0.87$-fold that of sham, $p < 0.05$) (Fig. 1D, E). Simultaneously, the qRT-PCR results revealed that VCR also induced a noticeable increase in the mRNA levels of inflammatory factors (TNF-$\alpha$, IL-6, IL-1$\beta$) and chemokines (CXCL12) in the spinal cord on days 3, 7, 14, and 21 compared to saline treatment (Fig. 1F-I). To verify whether VCR induces an apoptotic response, TUNEL staining was applied to evaluate the apoptotic response in the mouse spinal cord on day 7. The results confirmed that, compared with rare TUNEL-positive cells in the sham group, VCR led to a distinctly increased number of TUNEL-positive cells ($5.08 \pm 0.4$-fold of sham, $p < 0.05$) (Fig. 1J, K).

### 3.2 Cx43 plays a critical role in VCR-induced pain hypersensitivity by regulating the release of inflammatory factors and chemokines in spinal astrocytes.

Existing research has reported that Cx43 is the major component of intercellular connections in astrocytes (Wu et al. 2012). We detected Cx43 protein expression in the spinal cord at days 3, 7, 14, and 21 by Western blotting analysis. Compared with saline-treated animals at the same time points, VCR led to profound upregulation of Cx43 levels at all the time points examined (Fig. 2A, B). We next examined whether Cx43 played a crucial role in VCR-induced pain hypersensitivity in mice. First, we generated mice in which spinal Cx43 expression was focally knocked down through intrathecal administration of an antisense siRNA specifically targeting Cx43 mRNA (5 µg, 10 µl) on day six and day 7. As shown in Fig. 2C and D, intrathecal injection of Cx43 siRNA but not with a missense siRNA injection significantly improved VCR-induced pain hypersensitivity 36 h after injection. Western blotting analysis and qRT-PCR suggested that the Cx43 level in the spinal cord was markedly decreased after genetic knockdown of Cx43 using Cx43 siRNA (Fig. 2E-G).

To provide further evidence supporting the role of spinal Cx43 in VCR-induced pain hypersensitivity, we tested the effects of Gap27, a specific inhibitor of Cx43. We first injected Gap27 (144 nM, 10 µl) intrathecally into saline-treated mice to rule out the impact of Gap27 on the mechanical and thermal pain
threshold of normal mice (Supplement 1A, B). Subsequently, we treated mice exhibiting pain hypersensitivity symptoms with Gap27 on day 6 and day 7. The results indicated that intrathecal injections of Gap27 (14.4, 43.2, 144 nM, 10 µl each) dose-dependently reversed mechanical allodynia and heat hyperalgesia 2 h after the injection (Fig. 3A, B). Western blotting analysis and qRT-PCR suggested that the protein and mRNA levels of Cx43 declined remarkably after the first intrathecal injection of Gap27 for 36 h (Fig. 3C-E). However, as shown in Fig. 3F and G, VCR-induced astrocyte activation did not improve after the downregulation of Cx43 expression by Gap27. Then, we tested the effect of Gap27 on inflammatory factors. The ELISA results demonstrated that VCR-induced upregulation of IL-1β and IL-6 secretion in serum was strikingly reduced by 38.40% and 24.53%, respectively, after Gap27 treatment (Fig. 3H, I). However, qRT-PCR confirmed that Gap27 did not obviously decrease the mRNA levels of inflammatory factors and chemokines (Fig. 3J). These results verified that Cx43 played a critical role in VCR-induced pain hypersensitivity by regulating the release of inflammatory factors and chemokines in astrocytes. We also compared the effects of the preventive administration of Gap27 (day 1) and therapeutic administration of Gap27 (day 6 and day 7) on pain hypersensitivity. Compared with therapeutic administration of Gap27, preventive administration of Gap27 could also prevent pain hypersensitivity caused by VCR and had a low frequency of administration and a long duration of efficacy (Fig. 3K, L). Western blotting analysis revealed that, compared to VCR group, Cx43 expression was reduced significantly on day three and day seven after preventive treatment with Gap27 (Fig. 3M, N). Therefore, we believe that prophylactic administration of Gap27 is likely more effective than therapeutic administration.

### 3.3 CaMKII and Cav3.2 are involved in VCR-induced [Ca^{2+}]_{i} upregulation and pain hypersensitivity

A growing number of studies have found that calcium ions are closely related to chemotherapy-induced pain hypersensitivity (Li et al. 2020, Siau and Bennett 2006). We investigated the role of intracellular Ca^{2+} in pain hypersensitivity. To determine whether VCR stimulated the [Ca^{2+}]_{i} increase in primary astrocytes, the Ca^{2+} indicator Fluo-4/AM was used to measure intracellular Ca^{2+}. The results suggested that VCR (3 nM) rapidly stimulated an increase in [Ca^{2+}]_{i} in both normal physiological buffer and Ca^{2+}-free physiological buffer, and the increase in [Ca^{2+}]_{i} in Ca^{2+}-free physiological buffer was slightly smaller than that in normal physiological buffer (Fig. 4A). Therefore, the [Ca^{2+}]_{i} accumulation sources may be composed of the intracellular store's release and extracellular fluid, and the former occupied a larger proportion. Earlier, we mentioned that CaMKII and Cav3.2 are involved in the regulation of Ca^{2+}. In our research, we detected that the VCR-stimulated [Ca^{2+}]_{i} increase was significantly inhibited by pretreatment with KN-93 (10 µM), but not by L-Ascorbic acid (500 µM), for 1 h in Ca^{2+}-free physiological buffer; however, after adding Ca^{2+} to Ca^{2+}-free physiological buffer, [Ca^{2+}]_{i} increased rapidly in the KN-93 group, while the change in the L-Ascorbic acid group was not noticeable (Fig. 4B). An increase in [Ca^{2+}]_{i} was also inhibited after pretreatment with both KN-93 and L-Ascorbic acid in normal physiological buffer (Fig. 4C).
These results suggested that VCR stimulates an $[\text{Ca}^{2+}]_i$ increase via intracellular CaMKII-sensitive $\text{Ca}^{2+}$ stores, and Cav3.2 could regulate the influx of $\text{Ca}^{2+}$ in the extracellular fluid. Therefore, CaMKII and Cav3.2 were involved in VCR-induced $[\text{Ca}^{2+}]_i$ upregulation in astrocyte cultures.

To characterize the role of CaMKII and Cav3.2 in VCR-induced pain hypersensitivity, mice were injected intrathecally with KN-93 (70 nM, 10 µl) and L-Ascorbic acid (30, 100, 300 µM; 10 µl each) on day 6 and day 7 after eliminating the influence of the inhibitors KN-93 and L-Ascorbic acid on the mechanical and thermal pain threshold of mice (Supplement 1C-F). Behaviour was assessed at 2 h after administration, and the results showed that KN-93 relieved mechanical allodynia and heat hyperalgesia, and this effect was maintained for at least 48 h (Fig. 4D, E). Intrathecal injections of L-Ascorbic acid rapidly (2 h) reversed VCR-induced pain hypersensitivity in a dose-dependent manner (Fig. 4G, H).

### 3.4 CaMKII and Cav3.2 participate in VCR-induced pain hypersensitivity by activating astrocytes and increasing Cx43 expression.

To further verify the role of CaMKII in VCR-induced pain hypersensitivity, we examined protein changes in CaMKII. We examined phosphorylated CaMKII (p-CaMKII, an active state of CaMKII) in the spinal cord on days 3, 7, 14, and 21 by Western blotting analysis. The data revealed that VCR led to an obvious upregulation in p-CaMKII protein levels at all the time points examined but did not affect CaMKII expression (Fig. 5A, B). Previous studies have proven that astrocyte activation is associated with calcium ion flux in chemotherapy-induced pain hypersensitivity (Sompol and Norris 2018, Zamora et al. 2020). In this study, KN-93 and L-Ascorbic acid inhibited the VCR-stimulated $[\text{Ca}^{2+}]_i$ increase. We wondered whether spinal CaMKII and Cav3.2 participate in the development of pain hypersensitivity by promoting astrocyte activation. Mice were injected intrathecally with KN-93 (70 nM, 10 µl) or L-Ascorbic acid (300 µM, 10 µl) separately on two successive days, and then various biochemical indicators were tested. Confocal images of spinal sections co-stained with p-CaMKII with GFAP demonstrated that the VCR-induced increase in GFAP-positive astrocytes and p-CaMKII was significantly inhibited in mice injected with KN-93 intrathecally (Fig. 5C-E). Western blotting analysis further showed that VCR-induced CaMKII activation was inhibited by KN-93 (Fig. 5F, G). In addition, KN-93 also decreased the protein and mRNA levels of Cav3.2 and Cx43 (Fig. 5F, H-K). Intrathecal injection of L-Ascorbic acid (144 nM, 10 µl) also inhibited the activation of astrocytes (Fig. 6A, B) and reduced the levels of Cav3.2, p-CaMKII, and Cx43 (Fig. 6A, C-I). However, intrathecal injection of Gap27 did not affect the protein levels of p-CaMKII or Cav3.2 (Fig. 6J-L). These results verified that CaMKII and Cav3.2 might coregulate the expression of Cx43 through astrocyte activation. Moreover, CaMKII and Cav3.2 may have a mutual regulatory effect, which is probably related to their ability to regulate calcium ions. Therefore, CaMKII and Cav3.2 play essential roles in VCR-induced pain hypersensitivity.
3.5 The CaMKII signalling pathway is involved in the inflammation and apoptosis caused by VCR.

Our previous research examined that VCR could cause an inflammatory response (Zhou et al. 2018). Therefore, we surveyed the influence of the CaMKII signalling pathway on inflammation in the present experimental setting. First, we examined the expression of NF-κB pathway-related proteins in the spinal cord. Western blotting confirmed that KN-93 suppressed phospho-NF-κB p65 protein levels, but there was no difference in NF-κB p65 expression compared with sham treatment (Fig. 7A-B). Furthermore, KN-93 also inhibited the VCR-induced increase in COX-2 expression (Fig. 7A, D). Subsequently, we tested the changes in inflammation and chemokines in the spinal cord after inhibiting CaMKII phosphorylation. The qRT-PCR results suggested that intrathecal injection of KN-93 attenuated the increase in TNF-α, IL-6, IL-1β, and CXCL12 mRNA levels induced by VCR (Fig. 7E).

In this study, we found that VCR could cause an apoptotic response in the spinal cord. Therefore, we verified whether the CaMKII signalling pathway was related to the apoptotic response induced by VCR. TUNEL staining proved that the number of TUNEL-positive cells was significantly decreased by 79.02% following KN-93 treatment administration (Fig. 7F, G). Moreover, we tested the expression of apoptosis-associated proteins cleaved caspase-3, Bcl-2, and Bax by Western blotting and revealed that KN-93 strikingly reversed the dysregulation of cleaved caspase-3, Bcl-2, and Bax (Fig. 7H-N). Consistently, qRT-PCR demonstrated that KN-93 could improve the changes in Bcl-2 and Bax mRNA levels (Fig. 7O, P). These results indicated that the CaMKII signalling pathway participated in VCR-induced inflammation and apoptosis.

3.6 VCR induces a significant increase in Cx43 expression in vitro

To check the cytotoxicity of VCR, cell viability was measured in cultured primary astrocytes by a CCK-8 kit. After 24 h of treatment with VCR at a concentration that was increased from 0.1 nM to 100 nM, we discovered that VCR concentrations within 3 nM were safe for cells (Fig. 8A). We then performed immunohistochemical staining, Western blotting analysis, and qRT-PCR to confirm the effect of VCR on Cx43 expression. Confocal laser images showed that VCR treatment (3 nM, 24 h at 37 °C) evoked a pronounced upregulation in Cx43 levels in astrocyte cultures treated with VCR (Fig. 8B, C). In agreement with in vivo studies, the Western blotting analysis results proved that the expression of Cx43 was increased, and these increases were abolished by 41.22% in astrocyte cultures treated with Gap27 (30 µM) for 24 h (Fig. 8B-E). For the inflammatory response, the ELISA results suggested that inhibiting Cx43 could reduce the release of IL-6 and IL-1β in the cell supernatant (Fig. 8F, G). In addition, Cx43 siRNA was also used to treat astrocyte cultures. Following Cx43 siRNA treatment (1 mg/ml, 36 h), the expression of Cx43 was reduced by 55.09% in astrocyte cultures compared with missense siRNA treatment (Fig. 8H, I). Moreover, treatment with Cx43 siRNA decreased the release of IL-6 and IL-1β in the cell supernatant (Fig. 8J, K).
3.7 CaMKII and Cav3.2 regulate the expression of Cx43 in vitro

To further test the changes in CaMKII, Cav3.2 and Cx43 and their relationship, we pretreated astrocyte cultures with CaMKII, Cav3.2 and the Cx43 inhibitors KN-93 (10 µM), L-Ascorbic acid (500 µM) and Gap27 (30 µM) for 6 h and then treated them with VCR for 24 h. The Western blotting analysis results demonstrated that both KN-93 and L-Ascorbic acid inhibited CaMKII phosphorylation and downregulated the expression of Cav3.2 and Cx43 (Fig. 9A-D, G-J). qRT-PCR verified that KN-93 and L-Ascorbic acid reduced the mRNA levels of Cav3.2 and Cx43 (Fig. 9E, F, K, L). However, Gap27 only inhibited Cx43 protein and mRNA levels and did not affect CaMKII phosphorylation or Cav3.2 expression (Fig. 9M-O). In addition, we treated astrocyte cultures with the Cx43 plasmid to induce CX43 overexpression. The results of Western blotting analysis showed that the upregulation of Cx43 expression did not affect CaMKII phosphorylation or Cav3.2 levels (Fig. 9G, H). These results indicated that VCR was capable of activating the CaMKII signalling pathway and increasing Cav3.2 expression. Thus, CaMKII and Cav3.2 may interact and jointly regulate the expression of Cx43 in astrocyte cultures.

3.8. The CaMKII signalling pathway is associated with inflammation, mitochondrial injury and apoptosis in astrocyte cultures treated with VCR.

To clarify the potential mechanism of CaMKII involved in regulating the VCR-induced inflammatory response, the protein levels of P-p65 and p65 were detected by immunoblotting in primary astrocytes. Compared with the control group, P-p65 but not p65 protein levels were apparently upregulated in VCR-treated cells and were significantly blocked by KN-93 treatment (Fig. 10A-C). Then, we examined the mRNA levels of TNF-α, IL-6, IL-1β, COX-2, and CXCL12 by qRT-PCR and found that VCR-induced increases in inflammatory cytokines and chemokines were inhibited by KN-93 in astrocyte cultures (Fig. 10D).

Mitochondria play crucial roles in inflammation, immunity, autophagy, and cell death. Recent studies have confirmed that VCR can lead to mitochondrial functional damage (Chen et al. 2020). We wanted to know whether the CaMKII signalling pathway participated in mitochondrial damage caused by VCR. To evaluate the effect of CaMKII on VCR-induced mitochondrial damage, flow cytometry was performed to examine the mitochondrial membrane potential (MMP) when primary astrocytes were challenged with VCR and KN-93. As shown in Fig. 10E and F, VCR exposure reduced MMP, which was significantly alleviated by KN-93. We also tested the oxidative stress of primary astrocytes, and the data proved that KN-93 largely blocked ROS production induced by VCR (Fig. 10G, H). For the apoptotic response, the results of flow cytometry revealed that the VCR-triggered increase in apoptosis was remarkably reversed by KN-93 (Fig. 10I, J). Western blotting analysis also demonstrated that VCR-treated primary astrocytes significantly increased their protein expression of cleaved caspase-3 and Bax and reduced Bcl-2 expression, and these effects were reversed when they were exposed to KN-93 (Fig. 10K-Q). Taken together, the evidence above confirmed that the CaMKII signalling pathway was involved in the VCR-induced inflammatory response, mitochondrial damage, and apoptosis.
**Discussion**

Several studies have verified that astrocytes play a vital role in developing and maintaining chronic pain in the CNS (Sáez and Green 2018, Orellana, Martínez and Retamal 2013). The activation of astrocytes requires the participation of Ca\(^{2+}\) (Surmeier et al. 2010, Neher and Sakaba 2008). Therefore, we focused on CaMKII and Cav3.2 related to calcium ion regulation. Moreover, astrocytes form interconnected networks coupled through Cx43 (Bennett et al. 2012). Finally, our data suggested that CaMKII and Cav3.2 activate astrocytes by upregulating [Ca\(^{2+}\)], thereby modulating Cx43-dependent inflammatory factor release in the spinal cord of mice treated with VCR. Moreover, CaMKII was also involved in VCR-induced inflammation and apoptosis.

Chemotherapy-induced peripheral nerve damage could lead to astrocyte activation in the spinal dorsal horn (Zhou et al. 2020). Astrocyte activation is characterized by hypertrophy accompanied by an increase in GFAP, and its expression may be associated with proinflammatory cytokines (Sticozzi et al. 2020). Moreover, astrocyte activation promoted the development of CIPN by secreting regulatory factors, such as chemokines, pro-/anti-inflammatory factors and ATP (Shen et al. 2015, Dosch et al. 2019). In our research, we confirmed that VCR could persistently activate astrocytes and increase the production of inflammatory factors (TNF-\(\alpha\), IL-6, IL-1\(\beta\), and COX-2) and chemokines (CXCL12) from day 3 to day 21 after the first injection of VCR. In addition, VCR led to an apoptotic response in the mouse spinal cord.

Accumulating evidence suggests that astrocyte activation is related to calcium imbalance (Sompol and Norris 2018, Zamora et al. 2020). CaMKII is a widely expressed multifunctional serine/threonine kinase and plays a critical role in linking astrocyte activation and calcium dysregulation in age-related neurodegenerative diseases (Sompol and Norris 2018). In addition, CaMKII has been verified to participate in the survival and apoptosis of neuronal cells through Ca\(^{2+}\) signalling (Chen et al. 2010). Because CaMKII acts as a general integrator of Ca\(^{2+}\) signalling, we surmised that VCR likely activates astrocytes by promoting the phosphorylation of Ca\(^{2+}\)-dependent CaMKII and increasing [Ca\(^{2+}\)], in astrocytes. Indeed, our study showed that VCR not only caused [Ca\(^{2+}\)], disturbance in astrocytes but also increased the phosphorylation level of CaMKII. Blocking CaMKII phosphorylation effectively reversed the VCR-induced increase in [Ca\(^{2+}\)], and pain hypersensitivity induced by treatment with KN-93. Cav3.2, a low-threshold calcium channel, has been reported to be involved in regulating DRG neuronal excitability (McCallum et al. 2003) and is a molecular substrate for dorsal horn neuropathic pain (François et al. 2015). In this experiment, we also discovered that Cav3.2 controlled extracellular calcium entry into the cytoplasm in astrocytes treated with VCR. Similarly, after using L-Ascorbic acid to inhibit Cav3.2 expression, the VCR-induced [Ca\(^{2+}\)], imbalance and pain hypersensitivity were also improved. Moreover, inhibiting CaMKII phosphorylation and Cav3.2 expression can significantly reduce astrocyte activation and upregulate Cx43 levels. Thus, we believe that CaMKII and Cav3.2 likely activate astrocytes by upregulating [Ca\(^{2+}\)], in VCR-induced neuropathic pain. Existing evidence has revealed that CaMKII regulates Cav3.2 expression to enhance neuronal excitability (Welsby et al. 2003). However, we found
that CaMKII and Cav3.2 may interact because KN-93 or L-Ascorbic acid decreased the protein levels of CaMKII and Cav3.2 in this research.

Neighbouring astrocytes can communicate directly through intercellular gap junctions (Orellana et al. 2013). In addition, gap junction proteins also form unopposed hemichannels, which provide channels for cells to exchange molecules such as ATP to coordinate activities between the cytoplasmic and extracellular compartments (Retamal et al. 2007, Dosch et al. 2019). Connexin 43 (Cx43) is a significant component of intercellular connections in astrocytes. Increased expression of Cx43 was reported in the spinal cord following paclitaxel-induced and oxaliplatin-induced peripheral neuropathy, bone cancer pain (BCP), and chronic constriction injury (CCI) (Dai et al. 2020, Yang et al. 2018, Tonkin et al. 2018). Cx43 also modulates the astrocytic release of CXCL1, and CXCL1 maintains late-phase neuropathic pain by activating CXCR2 receptors in dorsal horn neurons in the spinal cord (Chen et al. 2014). However, a previous paper confirmed that the downregulation of spinal Cx43 expression by intrathecal treatment with Cx43-targeting siRNA increased IL-6 and COX-2 expression and induced hind paw mechanical hypersensitivity (Morioka et al. 2018). In our present research, we found that VCR could induce continuous upregulation of the Cx43 protein from day 3 to day 21 and increase the release of IL-6 and IL-1β in the spinal cord. The expression of Cx43 was significantly suppressed by intrathecal injection of the Cx43 inhibitors Gap27 and siRNA, which reduced the release of IL-6 and IL-1β and relieved VCR-induced pain hypersensitivity. However, Gap27 did not improve VCR-induced astrocyte activation or the production of inflammatory cytokines and chemokines in the spinal cord. Gap27 also did not reverse the increase in CaMKII and Cav3.2 expression induced by VCR. Likewise, astrocytes treated with Gap27 or Cx43 siRNA also exhibited decreased Cx43 expression and inflammatory factor release. In addition, we treated astrocyte cultures with a Cx43 plasmid to induce CX43 overexpression, and the results revealed that the upregulation of Cx43 expression also did not affect CaMKII phosphorylation or Cav3.2 levels. Therefore, CaMKII and Cav3.2 mediate Cx43-dependent inflammation by activating astrocytes in VCR-induced neuropathic pain.

In this experiment, we discovered that VCR (3 nM) stimulated an increase in [Ca^{2+}]_{i} in both normal physiological buffer and Ca^{2+}-free physiological buffer. Hence, the [Ca^{2+}]_{i} accumulation sources may include extracellular fluid influx and intracellular store release, and intracellular store release provided a larger proportion of [Ca^{2+}]_{i}. Subsequently, we pretreated astrocytes with KN-93 and L-Ascorbic acid for 1 h in Ca^{2+}-free physiological buffer. The results suggested that KN-93, not L-Ascorbic acid, effectively inhibited the release of Ca^{2+} from intracellular stores. Therefore, we believe that the CaMKII signalling pathway is essential for VCR-induced neuropathic pain. Recent studies have shown that reducing the phosphorylation of CaMKII inhibits NF-κB signalling pathway activation and diminishes lipopolysaccharide-induced neuroinflammation in primary microglial cells (Park et al. 2020). During our research, we noticed that when KN-93-pretreated primary astrocytes were treated with VCR for 24 h, the P-p65 level was prominently decreased compared to that of vehicle-pretreated cells. In vivo experiments also showed the same results. Therefore, CaMKII may participate in VCR-induced neuroinflammation by inhibiting NF-κB signalling pathway activation. For VCR-induced apoptosis, a question is how CaMKII
governed this process. A growing number of studies have investigated whether the interaction between ROS and cellular Ca$^{2+}$ activates related signalling pathways, leading to neuronal apoptosis (Circu, Aw and medicine 2010, Cheng et al. 2012a). Moreover, mitochondria play a crucial role in cellular Ca$^{2+}$ and redox homeostasis and apoptosis induction (Cheng et al. 2012a, Koopman et al. 2010). Oxidation can damage mitochondrial function, resulting in hindered energy regulation (Zsurka and Kunz 2015). In our experiments, we observed that VCR induced apoptosis-associated protein (cleaved caspase-3, Bcl-2, and Bax) dysregulation and increased the cell apoptosis rate, and VCR led to mitochondrial damage by increasing ROS production and changing MMP in astrocyte cultures. These disorders were reversed by the CaMKII phosphatase inhibitor KN-93. Thus, we tentatively conclude that CaMKII may participate in VCR-induced apoptosis by regulating the crosstalk between Ca$^{2+}$ signalling and mitochondrial ROS. Undoubtedly, more experiments are necessary to determine this issue.

In conclusion, we provide evidence that CaMKII, Cav3.2, and Cx43 play vital roles in neuropathic pain and reveal that CaMKII and Cav3.2 facilitate Cx43-mediated inflammatory factor release in spinal cord astrocytes to underlie the development of peripheral neuropathic allodynia. To the best of our knowledge, our study is the first report that CaMKII and Cav3.2 mediate Cx43-dependent inflammation by activating astrocytes in VCR-induced neuropathic pain. Furthermore, we confirmed that CaMKII was also involved in VCR-induced inflammation and apoptosis. These findings may have considerable benefit for the treatment of CIPN.

**Declarations**

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**Ethics statement:** The animal study was reviewed and approved by Institutional Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China; licence number: SYXX (Su) 2016-0011).

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and material:** All data generated or analysed during this study are included in this published article.

**Author Contributions:** Yunman Li and Yahui Hu developed the idea of the study, participated in its design and coordination, and revised the manuscript. Guizhou Li, Qingyan Yang and Yahui Hu performed the
experiments, analyzed data. Yini Lu drafted the manuscript. All authors read and approved the final manuscript.

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