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ORIGINAL ARTICLE

Gabapentinoid Insensitivity after Repeated Administration is Associated with Down-Regulation of the $\alpha_2\delta$-1 Subunit in Rats with Central Post-Stroke Pain Hypersensitivity

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Abstract The $\alpha_2\delta$-1 subunit of the voltage-gated Ca^{2+} channel (VGCC) is a molecular target of gabapentin (GBP), which has been used as a first-line drug for the relief of neuropathic pain. GBP exerts its anti-nociceptive effects by disrupting trafficking of the $\alpha_2\delta$-1 subunit to the presynaptic membrane, resulting in decreased neurotransmitter release. We previously showed that GBP has an anti-allodynic effect in the first two weeks; but this is followed by insensitivity in the later stage after repeated administration in a rat model of central post-stroke pain (CPSP) hypersensitivity induced by intra-thalamic hemorrhage. To explore the mechanisms underlying GBP insensitivity, the cellular localization and time-course of expression of the $\alpha_2\delta$-1 subunit in both the thalamus and spinal dorsal horn were studied in the same model. We found that the $\alpha_2\delta$-1 subunit was mostly localized in neurons, but not astrocytes and microglia. The level of $\alpha_2\delta$-1 protein increased in the first two weeks after injury but then decreased in the third week, when GBP insensitivity occurred. Furthermore, the $\alpha_2\delta$-1 down-regulation was likely caused by later neuronal loss in the injured thalamus through a mechanism other than apoptosis. In summary, the present results suggest that the GBP receptor $\alpha_2\delta$-1 is mainly expressed in thalamic neurons in which it is up-regulated in the early stage of CPSP but this is followed by dramatic down-regulation, which is likely associated with GBP insensitivity after long-term use.

Keywords Central post-stroke pain · Calcium channel $\alpha_2\delta$ subunit · Gabapentinoid · Thalamic hemorrhagic stroke · Thalamus · Spinal dorsal horn

Introduction

Central post-stroke pain (CPSP) is a type of central neuropathic pain that is induced by a primary lesion of the central somatosensory system following ischemic or hemorrhagic stroke [1–3]. CPSP occurs most often after strokes that involve the thalamus [4–9]. The quality of life of patients with CPSP is very poor due to daily paroxysms of persistent spontaneous pain and hypersensitivity to noxious (hyperalgesia and allodynia) and non-noxious stimuli (paresthesia and dysesthesia) [10–13]. So far, the clinical treatment of CPSP has been inadequate due to resistance to both drug and non-drug therapies in about half of the affected patients [1, 2, 14–17].

The gabapentinoids, including gabapentin (GBP) and pregabalin, are a class of anticonvulsants originally approved by the U.S. Food and Drug Administration (FDA) for the treatment of epilepsy. In 2004, gabapentinoids were also approved as first-line drugs for the treatment of some types of neuropathic pain, such as painful diabetic neuropathy, post-herpetic neuralgia, and spinal cord injury-induced pain [3, 14, 15, 18]. Nonetheless, gabapentinoids are commonly used to treat other types of

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neuropathic pain including CPSP, although their safety and efficacy have not yet been approved by the FDA. In a recent placebo-controlled trial, pregabalin failed to show significant improvement over placebo in patients with CPSP [19]. More recently, we evaluated the anti-allodynic effects of GBP in rats with CPSP hypersensitivity induced by experimental thalamic hemorrhage and found that, although GBP had a dose-dependent anti-allodynic effect following a single systemic administration, the effectiveness was transient, gradually decreasing after repeated administration for 14 days, implying the existence of drug insensitivity [20]. The GBP insensitivity was shown by a decreased maximal possible effect and the shortened time-course of a single administration at a lower dose (10 mg/kg, i.p.) or by a shortened time-course of a single administration at a higher dose (100 mg/kg, i.p.) [20]. These results are evidence of both spatial and temporal changes in the molecular targets of GBP in the CNS of these rats.

Pharmacologically, gabapentinoids have high affinity for the $\alpha_2\delta$-1 subunit of the voltage-gated Ca^{2+} channel (VGCC) in the CNS [21–23]. They inhibit presynaptic neurotransmitter release from hyperexcitable or abnormal neurons by blocking trafficking of the $\alpha_2\delta$-1 subunit to the presynaptic membrane [24–27]. Experimentally, expression of $\alpha_2\delta$-1, but not $\alpha_2\delta$-2 (another binding site of GBP), is significantly increased in both the dorsal root ganglia (DRG) and the dorsal horn of the spinal cord in animal models of peripheral neuropathic pain. This up-regulation is thought to contribute to the development of allodynia and hyperalgesia [28–31]. It has thus been proposed that gabapentinoids alleviate neuropathic pain by blocking the trafficking of $\alpha_2\delta$-1 to the presynaptic terminals of DRG neurons and this subsequently leads to reduced Ca^{2+} influx and neurotransmitter release in the spinal dorsal horn and the inhibition of central sensitization [27, 32].

Given that the $\alpha_2\delta$-1 subunit of the VGCC is the molecular target of GBP, we proposed that the protein level of this subunit would increase shortly after intra-thalamic hemorrhage during the development of post-stroke pain. We then postulated that subsequent apoptotic loss of neurons surrounding the lesion focus that occurs at least 21 days after the initial injury would lead to reduced $\alpha_2\delta$-1 subunit expression in the presynaptic terminals of excitatory interneurons, leading to reduced anti-allodynic effectiveness of the drug due to the loss of the GBP effect.

Thus, this study was designed to determine: (1) which cells (neurons, astrocytes, or microglia) express the $\alpha_2\delta$-1 subunit after intra-thalamic hemorrhagic injury; (2) the time-course of changes in the protein level of the $\alpha_2\delta$-1 subunit in rats with CPSP following intra-thalamic hemorrhagic injury; and (3) whether neuronal loss occurs in parallel with the changes in protein levels of the $\alpha_2\delta$-1 subunit in rats with CPSP after intra-thalamic hemorrhagic injury. Finally, by examining the dorsal horn of the spinal cord, determine whether parallel changes occur within the ascending somatosensory system.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 280–320 g were provided by the Laboratory Animal Center of the Fourth Military Medical University (FMMU). Rats were housed in a climate-controlled room (22–26 °C) under a 12 h/12 h light/dark cycle with access to food and water *ad libitum*. Somatic functional evaluations were carried out between 09:00 and 18:30. The rats were acclimated to test boxes for >30 min on each day before the first test. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996), and followed the ethical guidelines for pain research in conscious animals of the International Association for the Study of Pain. This study was approved by the Animal Care and Use Committee of FMMU. The number of animals used and their suffering were minimized. Animals were randomly divided into two groups for the establishment of the CPSP model with thalamic hemorrhage: (1) rats receiving intra-thalamic microinjection of saline (ITS) ($n = 24$); and (2) rats receiving intra-thalamic microinjection of collagenase (ITC) ($n = 42$).

Surgery

Surgery was performed as described previously [20, 33, 34]. Rats were anesthetized with chloralose (0.3 g/kg, i.p.), and then securely fixed in a stereotaxic instrument (Narishige Scientific Instrument Lab, Tokyo, Japan). After a midline incision, an opening was made in the right skull with a dental drill. Collagenase type IV (Sigma-Aldrich China, Shanghai) or saline was microinjected into the ventrobasal complex and posterior thalamic nucleus (stereotaxic coordinates: bregma −3.48 mm anteroposterior; 3.6 mm lateral to the midline, and 6.2 mm ventral to the brain surface) on the right side [35]. The needle on a 0.5-μL microinjection syringe filled with collagenase or saline was lowered into the region of interest, followed (5 min later) by slow ITC (0.025 IU collagenase dissolved in 0.25 μL saline) or ITS (0.25 μL saline) over a period of 10 min. The syringe was left in place for 5 min after each injection to prevent spread of the agent to the brain surface. Then the needle was slowly withdrawn, the skin closed using 4.0 sutures, and all rats were allowed to recover in individual cages for at least 7 days.

Measurement of Mechanical Pain Sensitivity

Mechanical sensitivity was evaluated with von Frey monofilaments as described previously [20, 36]. Rats were placed on the metal mesh floor of a plastic chamber and mechanical stimuli were applied using monofilaments with ascending bending forces of 0.8 g, 2–20 g at 2-g increments, and then 25, 30, 45, and 60 g. Each monofilament was applied 10 times (once every several seconds) to the plantar area of each hind-paw to induce a withdrawal reflex. The bending force of the monofilament able to elicit a 50% withdrawal response was expressed as the paw withdrawal mechanical threshold (PWMT, g).

Double Immunofluorescent Labeling

Double immunofluorescent labeling was performed to determine the cellular distribution of the VGCC $\alpha_2\delta$-1 subunit in the thalamus. The primary antibodies were mouse monoclonal anti-dihydropyridine receptor (anti-Cav$\alpha_2\delta$-1 subunit) (1:200, Novus Biologicals, Littleton, CO), rabbit anti-NeuN (1:200, abCam, Cambridge, UK), rabbit anti-GFAP (1:250, Millipore, Billerica, MA), and rabbit anti-Iba-1 (1:250, WAKO, Osaka, Japan). Secondary antibodies were FITC-conjugated goat anti-mouse IgG (1:200, Sigma, St. Louis, MO) and Cy3-conjugated sheep anti-rabbit IgG (1:200, Sigma). The animals were deeply anesthetized with urethane (2 g/kg, i.p.) on day 7 after intra-thalamic hemorrhage. They were perfused intracardially with saline followed by a phosphate-buffered solution of 4% paraformaldehyde. The brain was removed and post-fixed in the same fixative overnight, followed by cryoprotection in 30% phosphate-buffered sucrose. Transverse frozen sections (45 μm thick) were cut on a CM1900 freezing microtome (Leica, Wetzlar, Germany) and collected in 0.01 mol/L PBS. These sections were treated with Tris–HCl buffer and 3% H_2O_2 for 10 min to quench the endogenous peroxidase. Non-specific protein was blocked by incubation in PBS containing 1% bovine serum albumin (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 2 h at room temperature. Then the sections were incubated with the primary antibodies overnight at 4 °C. After three washes with PBS, the secondary antibodies were conjugated for 3 h at room temperature with agitation. Then the sections were rinsed, mounted on slides, and cover-slipped. Photomicrographs were obtained under a laser scanning confocal fluorescence microscope (Olympus FV1000, Tokyo, Japan) and processed with Image-Pro Plus digitizing software (Olympus). For details of the procedures see our previous reports [37, 38].

Western Blotting

The bilateral thalamus and the lumbar spinal cord (L3–L5) were obtained under deep anesthesia with urethane (2 g/kg, i.p.) on days 7, 14, and 21 after ITC or ITS, as in our previous report [20]. The tissues were homogenized in an ice-cold mixture of protease inhibitors and RIPA lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS) (Applygen Technologies Inc., Beijing, China). Total proteins were extracted by centrifugation at 12,000 g for 10 min at 4 °C. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA). The same amounts of proteins (60 μg) were heated for 10 min at 98 °C, separated on 10% SDS-PAGE by electrophoresis (Bio-Rad), and transferred onto PVDF membranes (Immobilon P, Millipore). The membranes were blocked with 5% skim milk for 3 h at room temperature and incubated with mouse monoclonal anti-dihydropyridine receptor (anti-$Ca_v\alpha_2\delta$-1 subunit) (1:200, Novus Biologicals) or mouse anti-β-tubulin antibody (1:20000, Sigma) overnight at 4 °C. The membranes were displayed with enhanced chemiluminescence reagents and images captured with FluorChem FC2 (Alpha Innotech Corp.). The density of the band area was quantified with AlphaImager software. All western blot analyses were performed at least three times from more than three rats.

Statistical Analysis

Data are expressed as mean ± SEM. The unpaired Student's *t* test was used for single comparisons. One-way ANOVA followed by the Dunnett's or Tukey's test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Establishment of a Rat Model of CPSP Hypersensitivity

Similar to our previous report [20] unilateral ITC injections confined to the medial lemniscus-ventrobasal complex-posterior thalamic nucleus (Fig. 1A) resulted in bilateral reductions of the PWMT, suggesting the occurrence of bilateral mechanical pain hypersensitivity after intra-thalamic hemorrhage (Fig. 1B). Out of the total 42 rats, 64.29% (27/42) displayed persistent bilateral mechanical hypersensitivity from day 7 to the end of behavioral measurements (Fig. 1B).

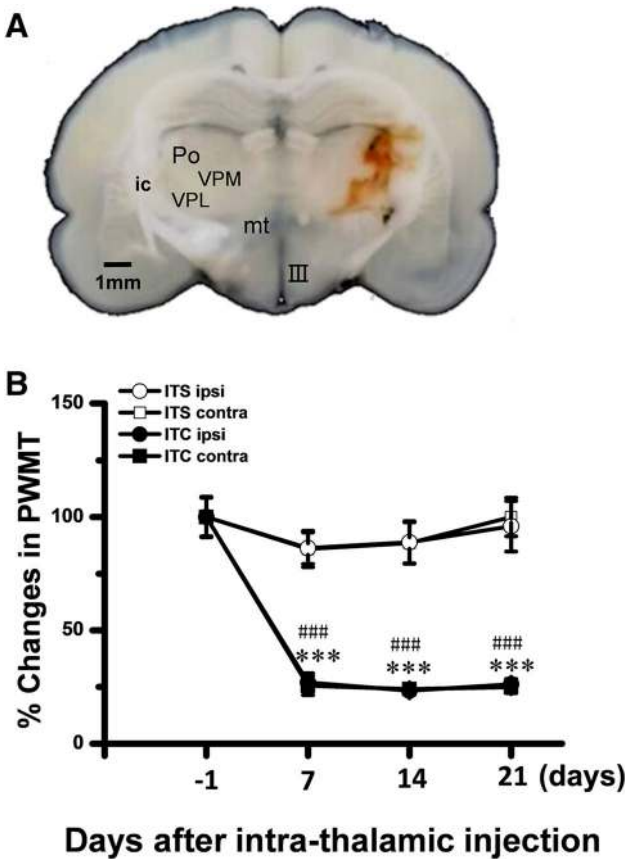

Fig. 1 Development of hemorrhagic central post-stroke pain hypersensitivity induced by intra-thalamic collagenase microinjection. **A** Photomicrograph of a 600-μm slice showing the hemorrhagic lesion site in the thalamus following unilateral intra-thalamic collagenase (ITC) microinjection. Scale bar, 1 mm. **B** Development of central post-stroke pain hypersensitivity to mechanical stimuli applied to both hind paws induced by ITC microinjection. Intra-thalamic saline (ITS) injection served as the control. III, third ventricle; contra, contralateral; ic, internal capsule; ipsi, ipsilateral; mt, mammillothalamic tract; Po, posterior thalamic nuclear group; PWMT, paw-withdrawal mechanical threshold; VPL, ventral posterolateral nucleus of the thalamus; VPM, ventral posteromedial nucleus of the thalamus. $^{***}P < 0.001$ ITC ipsi *vs* ITS ipsi; $^{###}P < 0.001$ ITC contra *vs* ITS contra, $n = 6$–8 animals/group.

Cellular Localization of $\alpha_2\delta$-1 Subunit in the Thalamus of Rats with CPSP Hypersensitivity

To identify the cellular localization of the $\alpha_2\delta$-1 subunit, sections containing both thalami were obtained from rats with CPSP hypersensitivity 7 days after ITC when GBP has a stable anti-allodynic effect as shown in our previous report [20]. The $\alpha_2\delta$-1 subunit was localized primarily in neurons surrounding the hemorrhagic focus (Fig. 2A). Labeling of astrocytes and microglia was also detected adjacent to the edge of the lesion (Fig. 2B2, C2). Double immunofluorescence labeling showed that the $\alpha_2\delta$-1 subunit mainly co-localized with NeuN (Fig. 2A and A'), but scarcely with GFAP and Iba-1 (Fig. 2B, B', C, C'), suggesting that the $\alpha_2\delta$-1 subunit is mainly expressed in neurons surrounding the hemorrhagic center.

Time-Course of $\alpha_2\delta$-1 Protein Expression in Rats with CPSP Hypersensitivity

Rats with CPSP hypersensitivity were randomly allowed to survive for 7, 14, and 21 days after ITC. At the diencephalic level, the $\alpha_2\delta$-1 protein was bilaterally up-regulated at 7 and 14 days after unilateral ITC injection (Fig. 3). However, the level of $\alpha_2\delta$-1 protein on day 21 post-injection was significantly lower bilaterally than in matched sites on days 7 and 14 (Fig. 3).

The expression of $\alpha_2\delta$-1 following unilateral ITC injection on days 7 and 14 was up-regulated bilaterally in the dorsal horn of the spinal cord when compared to the ITS controls (Fig. 4). Similar to the results in the thalamus, the $\alpha_2\delta$-1 protein level on day 21 was also lower than that on days 7 and 14 after ITC (Fig. 4).

Later Neuronal Loss in the Hemorrhagic Thalamus of Rats with CPSP Hypersensitivity

Because the $\alpha_2\delta$-1 subunit was mainly localized in thalamic neurons on the hemorrhagic side (Fig. 2), neuronal loss was suspected to account for the decreased labeling on day 21 post-ITC injection. Thus, the amount of NeuN protein, a neuronal marker, was assessed using Western blot. The amount of NeuN was lower on the lesioned side at 21 days than on day 7 post-injection (Fig. 5). Unlike $\alpha_2\delta$-1, NeuN remained unchanged in the contralateral side on day 21.

To determine whether the neuronal loss is caused by apoptosis, the caspase-3 levels were assessed. The caspase-3 protein level was significantly up-regulated on both sides of the thalamus 7 days after ITC, but returned to baseline by day 21 post-injection (Fig. 6).

Discussion

The major findings of the present study are as follows: (1) the VGCC $\alpha_2\delta$-1 subunit was expressed mainly in neuronal cell bodies, but not in glial cells (astrocytes and microglia), of the thalamus in rats with CPSP hypersensitivity; (2) the $\alpha_2\delta$-1 subunit was significantly up-regulated on both sides of the thalamus and the dorsal horn to much higher levels from day 7 up to day 14 after ITC injection in rats with CPSP hypersensitivity, but decreased on day 21 after ITC; and (3) the down-regulation of the $\alpha_2\delta$-1 subunit in both sides of the thalamus and dorsal horn 21 days after ITC was likely caused by the later neuronal loss on the injured side of the thalamus. However, the apoptotic process was

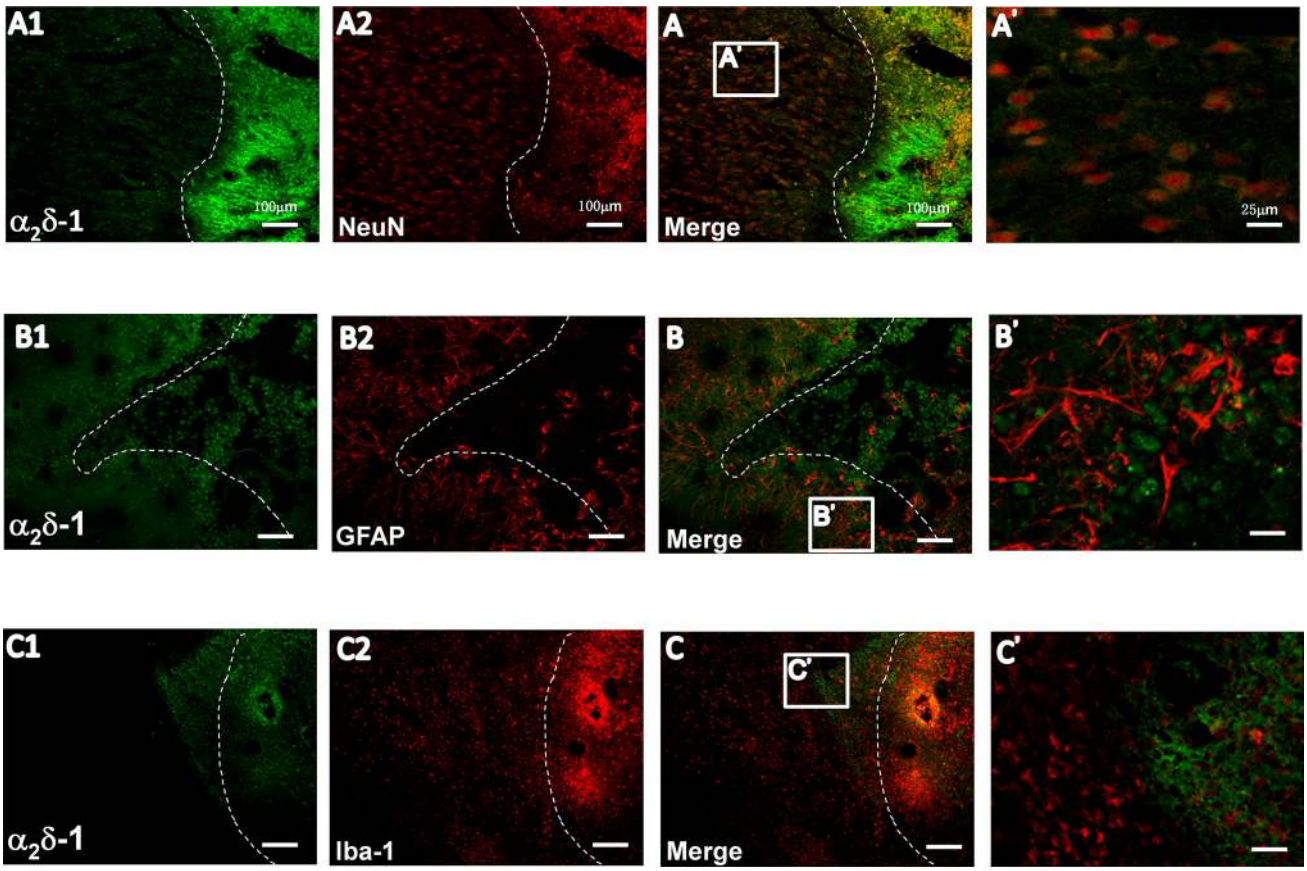

Fig. 2 Cellular localization of the $\alpha_2\delta$-1 subunit on the injured side of the thalamus after intra-thalamic hemorrhage. Laser confocal fluorescent images of double-staining for the $\alpha_2\delta$-1 subunit (**A1–C1**, *green*) with anti-NeuN, a marker for neurons (**A2**, *red*), anti-GFAP, a marker for astrocytes (**B2**, *red*), and anti-Iba-1, a marker for microglia (**C2**, *red*) on the injured side. **A–C** are merged images; **A'–C'** are magnifications of the boxes in **A–C**. *Dashed line* indicates border between the hemorrhagic injury center (*right*) and the adjacent surround (*left*). *Scale bars*, 100 μm for **A1–C1**, **A2–C2**, **A–C**; 25 μm for **A'–C'**.

probably not directly involved in the neuronal death because caspase-3 was activated on day 7 but not day 21 after ITC. This result is consistent with the original description of neuronal apoptosis in the same rat model of CPSP [34], which demonstrated that apoptosis reflected by TUNEL-positive cells is dramatic between 6 h and 3 days after ITC and declines significantly between 3 and 7 days after ITC, suggesting that apoptotic cell death is an early phenomenon following intra-thalamic hemorrhage [34]. Nonetheless, whether autophagic or secondary necrotic cell death is involved in this later neuronal death remains to be determined [39]. Taken together, expression of the $\alpha_2\delta$-1 subunit, a molecular target of GBP, is up-regulated in both the thalamus and dorsal horn in the first two weeks (7–14 days) after hemorrhagic stroke induced by ITC injection, while down-regulation of the $\alpha_2\delta$-1 subunit may begin sometime between days 7 and 21 after ITC due to the later neuronal loss. This is likely to be responsible for the decreased anti-allodynic effectiveness of GBP described in our recent report [20].

Gabapentinoid Insensitivity in Central Neuropathic Pain of Thalamic Hemorrhage

Gabapentinoids can effectively relieve many types of peripheral neuropathic pain in patients, including painful diabetic peripheral neuropathy [40, 41] and post-herpetic neuralgia [40, 42, 43]. GBP and pregabalin are also effective in relieving pain associated with spinal cord injury, a type of central neuropathic pain [44–46]. However, so far no evidence has been provided to support the use of gabapentinoids in the treatment of central neuropathic pain of thalamic damage caused by either ischemic or hemorrhagic stroke. In a 13-week, randomized, double-blind, multicenter, placebo-controlled trial, treatment with pregabalin did not provide significant pain relief in patients with CPSP [19].

This phenomenon has also been noted in animal studies. For instance, the anti-nociceptive and anti-allodynic effectiveness of GBP has been widely supported by studies in animal models of many types of pain induced by

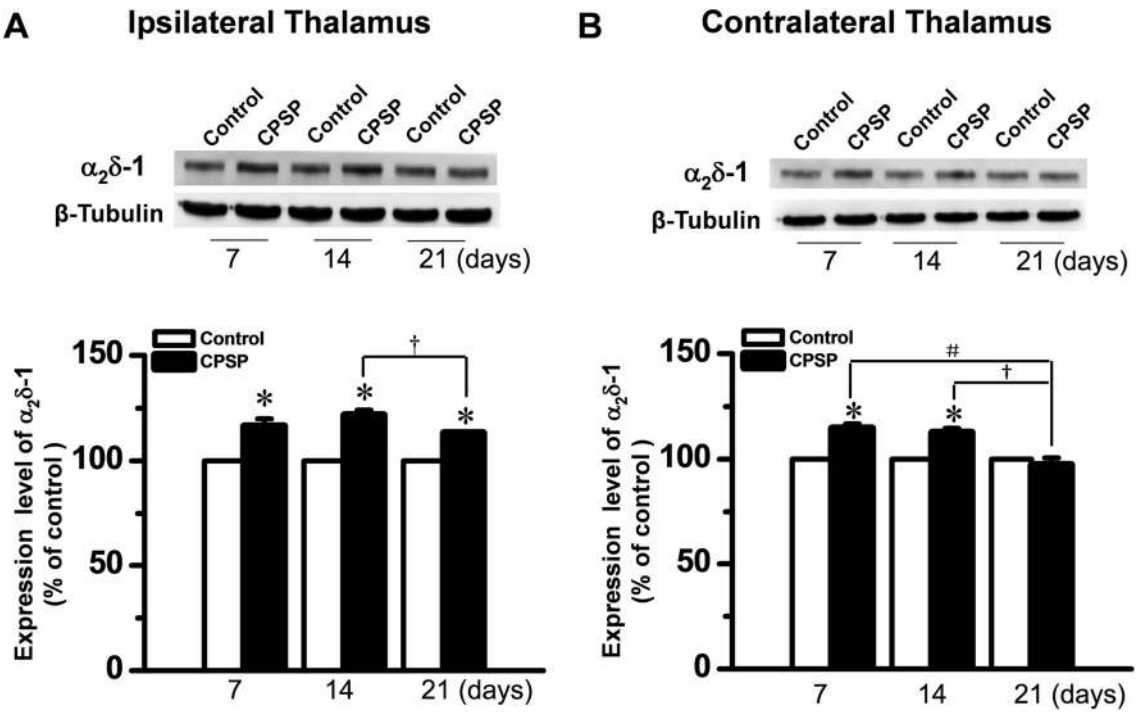

Fig. 3 Up-regulation of $\alpha_2\delta$-1 protein in bilateral thalami after unilateral intra-thalamic hemorrhage. **A**, **B** Upper panels: Western blots for time-related changes in $\alpha_2\delta$-1 protein levels in bilateral thalami 7, 14, and 21 days after unilateral intra-thalamic collagenase (ITC) microinjection. Lower panels: averaged data from 4–5 animals with central post-stroke pain (CPSP) hypersensitivity at the corresponding time points. Intra-thalamic saline (ITS) microinjection served as the control. $^{*}P < 0.05$ *vs* Control; $^{\#}P < 0.05$, $^{\dagger}P < 0.05$; $n = 4$/time-point.

peripheral nerve injury such as spinal nerve ligation [47–50], chronic compression injury [51–56] and spared nerve injury [57, 58]. Moreover, GBP has also been demonstrated to have anti-allodynic and anti-hyperalgesic effects in animal models of diabetic neuropathy [29] and post-herpetic neuralgia [59]. However, in our animal model of CPSP hypersensitivity, GBP delivered once daily *via* the intraperitoneal route only had a significant anti-allodynic effect during the first week of administration; GBP insensitivity occurred after the second week of treatment [20].

Taken together, it is likely that GBP is differentially effective in the relief of neuropathic pain of different origins. It may be effective in the treatment of peripheral neuropathic pain and spinal cord injury-associated pain, but may not be useful in the long-term treatment of central neuropathic pain due to thalamic hemorrhagic stroke.

Loss of the Gabapentin Effect May Contribute to Gabapentinoid Insensitivity in the Central Neuropathic Pain of Thalamic Hemorrhage

Both GBP and pregabalin are ligands of the VGCC $\alpha_2\delta$ subunit. It has been demonstrated that the VGCC $\alpha_2\delta$-1 subunit can be induced to overexpress in both DRG neurons and the dorsal horn in peripheral nerve injury models [26–28, 30, 60–62]. Overexpression of the $\alpha_2\delta$-1 subunit has been thought to contribute to the development of neuropathic pain hypersensitivity through its trafficking to the presynaptic membrane that leads to increased presynaptic Ca^{2+} influx and neurotransmitter release [24–27, 31, 32, 62–64]. The von Willebrand factor-A domain is involved in trafficking the VGCC $\alpha_2\delta$-1 subunit to the plasma membrane [23, 65, 66]. Knock-down of $\alpha_2\delta$-1 reduces pain hypersensitivity in an animal model of nerve injury [30]. It has also been confirmed that the anti-nociceptive and anti-allodynic effects of GBP and pregabalin are due to disruption of trafficking of the VGCC $\alpha_2\delta$-1 subunit to the presynaptic terminal membrane induced by nerve injury [25–27, 32]. Thus the pharmacological action of GBP against neuropathic pain could be dependent upon aberrant overexpression of the VGCC $\alpha_2\delta$-1 subunit in the presynaptic membrane. Based on the anti-allodynic mechanisms of GBP and the results of the present study, we propose that the onset of insensitivity of GBP later in the process of CPSP hypersensitivity is largely due to the loss of the GBP effect resulting from down-regulation of the $\alpha_2\delta$-1 subunit at both the thalamic and spinal levels of the somatosensory system.

However, other mechanisms of GBP insensitivity cannot be completely excluded because GBP insensitivity might

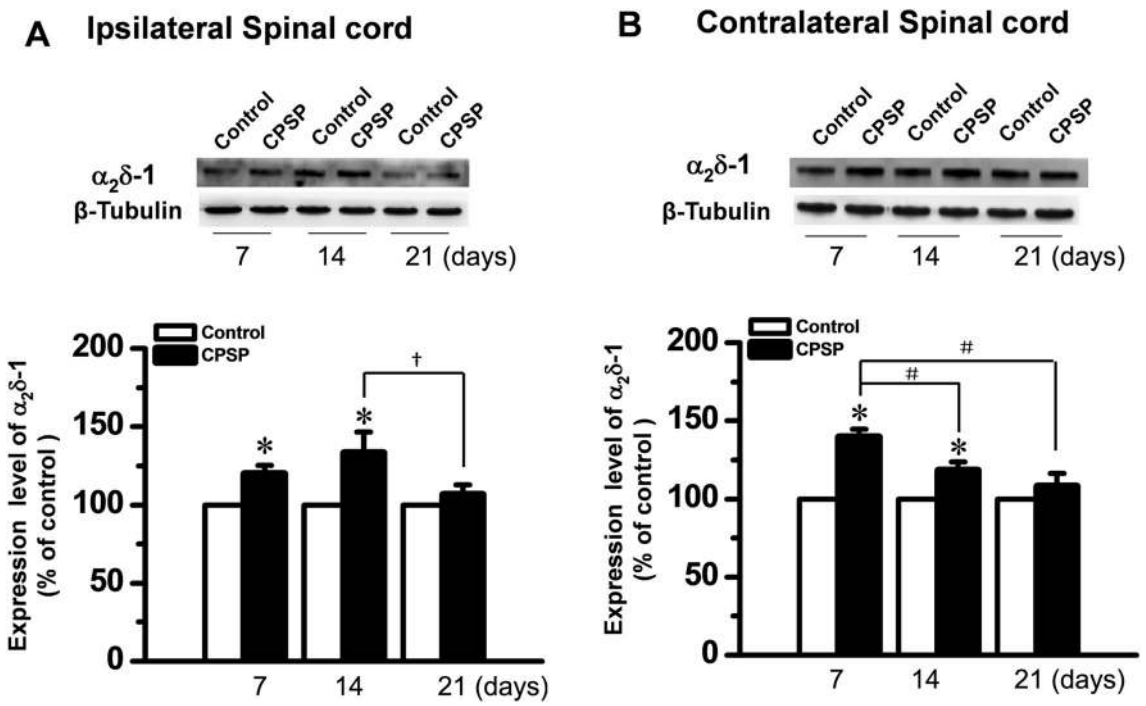

Fig. 4 Up-regulation of $\alpha_2\delta$-1 protein in bilateral spinal dorsal horn after unilateral intra-thalamic hemorrhage. **A**, **B** Upper panels: Western blots for time-related changes in $\alpha_2\delta$-1 protein levels in the bilateral dorsal horn 7, 14, and 21 days after unilateral intra-thalamic collagenase (ITC) microinjection. Lower panels: averaged data from 4–5 animals with central post-stroke pain (CPSP) hypersensitivity at the corresponding time points. Intra-thalamic saline (ITS) microinjection served as the control. $^{*}P < 0.05$ *vs* Control; $^{\#}P < 0.05$, $^{\dagger}P < 0.05$.

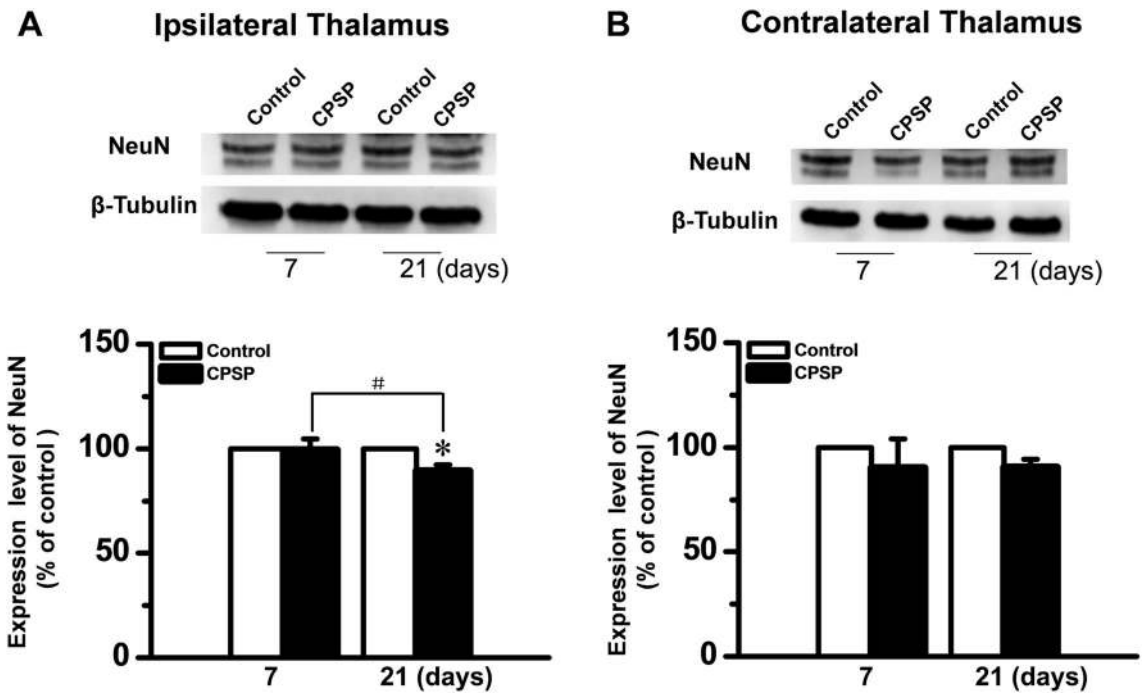

Fig. 5 NeuN protein levels in bilateral thalami after unilateral intra-thalamic hemorrhage. **A**, **B** Upper panels: Western blots for time-related changes in NeuN protein levels in bilateral thalami on days 7 and 21 after unilateral intra-thalamic collagenase (ITC) microinjection. Lower panels: averaged data from 3–4 animals with central post-stroke pain (CPSP) hypersensitivity at the corresponding time points. Intra-thalamic saline (ITS) microinjection served as the control. $^{*}P < 0.05$ *vs* Control; $^{\#}P < 0.05$.

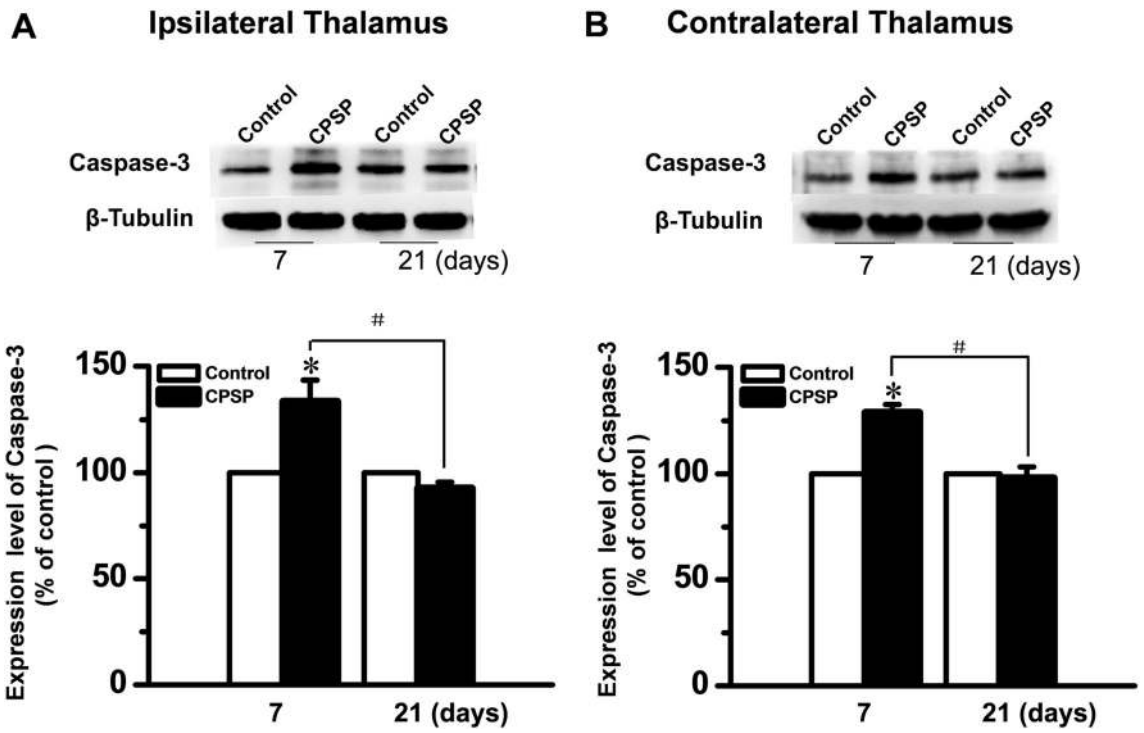

Fig. 6 Levels of caspase-3 protein, an effector of apoptosis, in bilateral thalami after unilateral intra-thalamic hemorrhage. **A**, **B** Upper panels: Western blots for time-related changes in caspase-3 protein levels in bilateral thalami on days 7 and 21 after unilateral intra-thalamic collagenase (ITC) microinjection. Lower panels: averaged data from 3–4 animals with central post-stroke pain (CPSP) hypersensitivity at the corresponding time points. Intra-thalamic saline (ITS) microinjection served as the control. $^{*}P < 0.05$ *vs* Control; $^{\#}P < 0.05$.

be due to changes in the $\alpha_2\delta$-1 expression level and the pharmacokinetic and pharmacodynamic characteristics in patients and animals with CPSP. As for the relationship between the level of $\alpha_2\delta$-1 expression and anti-allodynic effectiveness, it has been demonstrated that the effectiveness of a certain dose of GBP can change due to circadian changes in both the mRNA and the protein levels of $\alpha_2\delta$-1 in rats with partial sciatic nerve ligation (PSNL), and it was surprisingly noted that GBP sensitivity was augmented at a time when the $\alpha_2\delta$-1 subunit protein was lower at 05:00, while it was attenuated at a time when the $\alpha_2\delta$-1 subunit protein was abundant at 17:00 during 24 h of measurement [67]. Moreover, the maximal binding capacity of [^{3}H]-GBP in the ipsilateral DRG of rats with PSNL was also demonstrated to be two-fold higher at 17:00 than at 05:00 but with the affinity constant value unchanged [67]. However, the pharmacokinetic and pharmacodynamic characteristics of GBP in rats with PSNL does not change with the circadian rhythm [67]. Whether the levels of $\alpha_2\delta$-1 genes and proteins can be influenced by the circadian rhythm in rats with CPSP is not clear and remains to be further investigated. Nonetheless, because in our previous work [20] and the current study, the pharmacological experiments were performed between 09:00 and 15:00 and the perfusion of the animals subjected to immunoblotting was carried out between 15:00 and 17:00, our results were unlikely to be influenced by circadian effects. $\alpha_2\delta$-1 has also been demonstrated to be a thrombospondin (astrocyte-secreted protein) receptor in CNS neurons that is responsible for excitatory synaptogenesis. So, blocking the interaction between thrombospondin and $\alpha_2\delta$-1 by GBP could eliminate the thrombospondin-induced formation of excitatory synapses without affecting established synapses [68], and over-expression of $\alpha_2\delta$-1 in rats with CPSP might be involved in excitatory synaptogenesis in the thalamus and dorsal horn following thalamic hemorrhage. Long-term administration of GBP could play an inhibitory role in the thrombospondin-induced formation of excitatory synapses involved in the repair of thalamic hemorrhage-induced tissue damage; however, the thrombospondin-induced aberrant formation of excitatory synapses, if it occurs, is not likely to be involved in the late process of CPSP hypersensitivity due to the appearance of GBP insensitivity after repeated administration.

In conclusion, the GBP receptor $\alpha_2\delta$-1 is mainly expressed in thalamic neurons, in which it is up-regulated in the early process of CPSP but this is followed by dramatic down-regulation that is likely associated with GBP insensitivity after long-term use.

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