Distribution of TRPV1 in CSF Contacting Nucleus of Rat Brain Parenchyma and its expression in Neuropathic Pain

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Abstract

Background: The cerebrospinal fluid-contacting nucleus (CSF-CN), distributes and localizes in the ventral periaqueductual central gray (PAG) of the brainstem, which may influence actual composition of the cerebrospinal fluid (CSF) for non-synaptic signal transmission via releasing or absorbing bioactive substance. TRPV1 has been found in both the peripheral and spinal within centres known for their role in pain detection, transmission and regulation, consistent with its role in pain. Therefore, it is speculated that the CSF-CN participate in pain modulation via the receptor TRPV1. The present study aimed to observe the TRPV1 expression and distribution in the CSF-CN of rat brain parenchyma, and to explore the effects of TRPV1 on the CSF-CN in neuropathic pain.

Methods: The model of neuropathic pain with chronic constriction injury (CCI) of the sciatic nerve was made in Spague-Dawley rats. The thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were measured. The cholera toxin subunit B conjugated with horseradish peroxidase (CB-HRP) as a tracer was injected into one of the rats’ lateral ventricles (LV) to explore CSF-CN. The distribution and expression of TRPV1 were observed in the CSF-CN with double labeling of CB-HRP and TRPV1 with immunohistochemistry. A selective TRPV1 antagonist, SB-366791, was injected into one of the rat’s LV at the day of peak raise of allodynia and hyperalgesia (10 days after CCI surgery) in 5µg. The behavioral of rat were observed at 2h before and after administration.

Results: The CSF-CN is always located in a special region in rat brain parenchyma which is well consistent with the previous finding and found that most cells of CSF-CN have TRPV1. TRPV1 expression levels were significantly increased in the CB-HRP positive neurons at 10 days following CCI surgery. After intracerebroventricular administration of SB366791, mechanical allodynia and thermal hyperalgesia were attenuated at 5µg over a time period of approximately 4 hours, especially at 2h following administration.

Conclusion: TRPV1 is localized within the CSF-CN of the mesencephalon and its expression was increased in CSF-CN by CCI surgery. Comparative analgesic effects of a TRPV1 antagonist in a CCI model of neuropathic pain lend support to the validation of TRPV1 in CSF-CN as a promising target for the treatment of neuropathic pain.

Keywords: dCSF-CN; CSF-CN; TRPV1; SB366791; Neuropathic pain

Abbreviations: CB-HRP: Cholera toxin B conjugated to Horseradish Peroxidase; CSF-CN: Cerebrospinal Fluid-Contacting Neurons; dCSF-CN: distal CSF-CN; CSF-CN: Cerebral Spinal Fluid-Contacting Nucleus; IVC: Intracerebroventricular; LV: Lateral Ventricle; TRP: Transient Receptor Potential

Background

The TRPV1 channel, previously known as vanilloid receptor VR1, was cloned 10 years ago and is a calcium-permeable nonselective cation channel [1,2] that respond to mechanical, thermal, chemical (i.e. acid, lipids) and many other stimuli coming from the extra and intracelluar milieu [3-7]. It is expressed predominantly in nociceptors that participate in the transduction of noxious chemical and thermal stimuli in the peripheral nervous system (PNS). Bipolar neurons with unmyelinated axons (C fibers) and somata in dorsal root and trigeminal ganglia, as well as a subset of sensory neurons with thin myelinated axons (Aδ fibers), are capsaicin sensitive [8]. Currently, TRPV1 receptors are a novel therapeutic target in the PNS, and agonists and antagonists are being tested for the treatment of inflammatory and chronic neuropathic pain [9,10,11]. However, in neuropathic pain, in contrast to the well-established function of TRPV1 receptors in inflammatory pain, their roles are not well defined, especially in the central nervous system (CNS).

Recent studies have also shown that TRPV1 receptors are located in several regions of the CNS including hypothalamus, midbrain PAG, substantia nigra, and locus coeruleus [12,13,14]. Although there is evidence that TRPV1 receptors in the CNS are involved in pain transmission or modulation and might serve as useful drug targets [14-16], there is still existing controversial about their roles in the CNS. Activation of TRPV1 receptors induces hypalgesia by increasing glutamate release in substantia nigra, locus coeruleus, hypothalamus and PAG [17-19]. However, centrally penetrating TRPV1 receptor antagonists proved more effective compared to peripheral restricted agents with the same pharmacokinetic and pharmacological profile [20], underlining an involvement of central TRPV1 receptor blockade in the analgesic action. Nonetheless, the functional significance of TRPV1 receptor expression in the brain remains elusive.
Measurement of allodynia and hyperalgesic activity was performed in the same manner except for sciatic nerve ligation. With 1 mm space. The incision was closed in layers. Sham operation

nerve by using 4-0 braided silk thread (Ethicon Inc., Brussels, Belgium) at the level of the middle of the thigh by blunt dissection through biceps hydrate (300 mg/kg, i.p.), and the left common sciatic nerve was exposed and Xie (1998). Briefly, animals were anaesthetized with 10% chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with 150 ml of phosphate buffered saline (0.01 M PBS, pH 7.4), followed without interruption by 4% paraformaldehyde in 0.2 M phosphate buffer (300 ml, pH 7.4). The brainstem was removed immediately and post-fixed for 4-6 h at 4°C, then cryoprotected by immersion for 24-48 h in sucrose gradients (5%, 10%, 15%, and 26%) to the plantar surface of the hind paw. Each VFH was applied to the paw for 6s, or until a withdrawal response occurred. Once a withdrawal response was observed, the paw was re-tested, starting with the next descending VFH until no response was noted. The lowest amount of force required to elicit a response was recorded as the withdrawal threshold (PWT) in g. Static allodynia was defined as being present if the rats responded to the 2g VFH or below, which was innocuous to normal or sham-operated rats.

The method of Hargaves et al. (1988) was used to assess thermal withdrawal latency (TWL) to a thermal nociceptive stimulus. Rats were placed in a Plexiglas enclosure on top of the glass surface of a thermal testing apparatus (BME410A, Institute of Biological Medicine, Academy of Medical Science; China) and allowed to acclimate for 30 min before testing. A mobile radiant heat source (a high-intensity light beam) located under the glass was focused onto the hind paw. The TWL was recorded by a digital timer. Stimulus intensity was kept constant throughout the entire experiment, and was adjusted to give approximately a 10s TWL in the normal or sham operated hind paw. A cutoff time was set at 20s to avoid tissue damage. Rats were acclimated to the procedure until stable TWL values were obtained, and the mean TWL value of the last three measurements was used for analysis. A significant reduction in TWL compared with normal baseline was interpreted as thermal hyperalgesia. Rats not demonstrating hyperalgesia was not further studied (less than 5%).

After recording basal MWT and TWL at 10 days after CCI, SB366791 was administered. SB366791 was intracerebroventricularly administered at dose of 5ng (in 2µl solution). In the SB366791 study, 10% DMSO (2 µl) was used as a vehicle. To examine the effect of SB366791 on CCI rats, rats received CCI surgery without i.c.v. injecting SB366791 at 10 days as control. The sham group (sham operation) is SB366791 specifically against TRPV1 was dissolved in DMSO and was administrated 10 days after CCI by means of unilateral intracerebroventricular (i.c.v.) infusion 2h after recording basal MWT and TWL.

Methods

Animal experiments

Male Sprague-Dawley rats (250±50g) for experiments were obtained from the experimental animal center, Xuzhou Medical College. The number of the license is: SYXX (Jiangsu) 2002-0038, Grade SPF. Rats were maintained in climate and light-controlled (23±1°C, 12/12h dark/ light cycle with light on at 08:00h) for at least one week prior to the experiments. Efforts were made to minimize animal suffering and to reduce the number of animals used. Rats were used once only. All experiments were conducted in accordance with the guidelines of the International Association for the Study of Pain (IASP) and approved by the Committee for the Ethical Use of Laboratory Animals, Xuzhou Medical College.

Drug preparation

SB366791 (Enzo Life Sciences, San Diego, CA, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in physiological saline. The final DMSO concentration was 10%.

Chronic Constriction Injury (CCI)

The CCI was made according to the method described by Bennett and Xie (1998). Briefly, animals were anaesthetized with 10% chloral hydrate (300 mg/kg, i.p.), and the left common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatic’s trifurcation, about 7mm of nerve was freed of adhering tissue and 4 ligatures were loosely tied around the nerve by using 4-0 braided silk thread (Ethicon Inc., Brussels, Belgium) with 1 mm space. The incision was closed in layers. Sham operation was performed in the same manner except for sciatic nerve ligation.

Measurement of allodynia and hyperalgesic activity

The rats were habituated to grid bottom cages before the start of the experiment. The allodynia was evaluated by the application of von Frey hairs (VFHs; Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA) in ascending order of force (0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15 and 26g) to the plantar surface of the hind paw. Static allodynia was defined as being present if the rats responded to the 2g VFH or below, which was innocuous to normal or sham-operated rats.

The withdrawal response was observed, the paw was re-tested, starting with the next descending VFH until no response was noted. The lowest amount of force required to elicit a response was recorded as the withdrawal threshold (PWT) in g. Static allodynia was defined as being present if the rats responded to the 2g VFH or below, which was innocuous to normal or sham-operated rats.

Forty-eight h following tracer injection, rats were deeply anesthetized again with intraperitoneal 10% chloral hydrate (300 mg/ kg, i.p.) and transcardially perfused with 150 ml of phosphate buffered saline (0.01 M PBS, pH 7.4), followed without interruption by 4% paraformaldehyde in 0.2 M phosphate buffer (300 ml, pH 7.4). The brainstem was removed immediately and post-fixed for 4-6h at 4°C, then cryoprotected by immersion for 24-48 h in sucrose gradients (5%, 10%, 15%, 20%, and 30%) with 0.01 mol/L PBS at 4°C. The brainstem embedded with OCT, at -20°C and sectioned on a cryostat (Leica CM1900, Germany) at 40 µm in the transverse plane.
Immunofluorescence procedures and confocal microscopy technique

The frozen sections were collected in PBS. Following 3 washes in PBS, sections were incubated in PBS with 0.3% triton X-100 (PBST) for 48-72 h at 4°C with an goat anticholera toxin B-subunit in (1:500; 227040, Merck, Germany) and mouse Anti-TRPV1 (1:500; ab45759, Abcam, Cambridge, UK). After rinsing in PBS, sections were incubated and in donkey anti-goat IgG-conjugated to fluorescein isothiocyanate (1:400; ab6881, Abcam, Cambridge, UK), and in donkey anti-mouse IgG conjugated to Texas Red (1:400; ab7059, Abcam, Cambridge, UK), in the dark for 2 h at room temperature. Absence of immunostaining was also obtained by primary antibody omission (data not shown).

Finally, sections were rinsed, mounted, and coverslipped with glycerol containing 2.5% of anti-fading agent DABCO (1,4-di-aza-bi-cyclo-2,2,2-octane, Sigma, USA) and stored at -20°C in the dark. Tissue containing 2.5% of anti-fading agent DABCO (1,4-di-aza-bi-cyclo-2,2,2-octane, Sigma, USA) and stored at -20°C in the dark for 2 h at room temperature. Absence of immunostaining was also obtained by primary antibody omission (data not shown).

For the time course experiment, we measured the MWT and TWL 30 min before and 1 h, 2 h, 4 h, 8 h after the SB366791 administration. Results are expressed as mean ± S.D. (n = number of animals). All data were imported to Microsoft Excel 2007 (Microsoft co., Seattle, WA). All statistic tests were undertaken in SPSS v.16.0 (SPSS, Chicago, IL). The data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey’s t-test (or just a t-test where there were only two groups). P < 0.05 was considered to indicate statistical significance.

Results

Noxious behavioral response

Ligation of the common sciatic nerve produced a clear-cut hyperalgesia in rats submitted to the surgery compared to the sham operated rats (P < 0.01) (Figure 1). At 10 days after operation, CCI rats displayed mechanical allodynia and thermal hyperalgesia by the reduction in MWT and TWL to 4.86 ± 1.51 g and 9.32 ± 1.11 s compared to MWT of 16.98 ± 3.50 g and TWL of 16.93 ± 3.11 s in sham-operated rats. Intracerebroventricular administration of SB366791 (5µg), but not vehicle, reduced mechanical hyperalgesia and thermal hyperalgesia. Time to reach the maximal antihyperalgesic effect was 2 h after i.c.v. administering SB366791 and the effect decayed gradually in about 4 h (Figure 2). No changes in behavioral or motor function were observed in either group vehicle or sham.

Morphological and immunofluorescence characterization

TRPV1-expression for the CSF-CN: Positive labeling of the CB-HRP-traced neurons in the ventral aqueduct region was mainly located in cytoplasm, and the majorities were multipolar and round or oval in shape. The cell bodies of this dCSF-CNs were in the brain parenchyma and the processes extended into CSF. A tissue section, CB-HRP immunoreactivity in green FITC is illustrated in Figure 3A. TRPV1-positive neurons (Tex Red, red) were explained in Figure 3B. CB-HRP/ TRPV1 double-labeled neurons in yellow were shown in Figure 3C. An immunofluorescence analysis demonstrated the localization of TRPV1 in the CSF-CN.

Changes of TRPV1-expression in the CSF-CN after CCI: Positive neurons of CB-HRP, TRPV1 and CB-HRP/TRPV1 were existed both in CCI group and control group. The TRPV1-immunoreactive levels were

| Group | CB-HRP and TRPV1 double-labeled (yellow) | CB-HRP (green) | TRPV1 (red) | CB-HRP/ TRPV1 double-labeled/ CB-HRP (%) |
|-------|-----------------------------------------|----------------|-------------|---------------------------------------|
| 1 (control) | 111 ± 13 341a 38 256x22 32.7±3.2 |                |             |                                       |
| 2 (CCI) | 174 ± 15 340 ± 24 534±35 51.5±7.4 |                |             | All the brain tissue sections counted |
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leading to burning pain or itch mediated by discharges in C polymodal and Aδ mecano-heat nociceptors and in central neurons. The involvement of TRPV1 in inflammatory [32], neuropathic [33], and cancer pain [34] has been reported. This study is the first to our knowledge showing that TRPV1 is expressed in the distal CSF-contacting neurons of mesencephalon ventral to the cerebral aqueduct. This indicates that TRPV1 is expressed in CSF-CN under normal physiological conditions, suggesting that TRPV1 signaling pathways

higher in CCI group (Figure 4C) than in control rats (Figure 4A) at 10 days after operation. TRPV1-expressing in CSF-CN was significantly increased at 10 days after CCI. The number of CB-HRP/TRPV1 dual-labeled neurons in CCI group were 174±15, significantly higher than control group-111±13, which is positively relevant to the withdrawal behavior signs (P<0.01) (Figure 4E, Table 1).

Discussion

The dCSF-CNs, whose bodies are in the brain parenchyma and processes extend into CSF in the brain, may play an important role in the signaling, substance transport, functional modulating between the brain and CSF [22]. Both clinical practice and animal experiments indicate that the chemical composition of the CSF can change in pathological or special physiological conditions [21,22,24]. The reasons, origin and receptors for these chemical changes remain unclear. In the first time our study used CB-HRP as a tracer retrograde to display dCSF-CNs clearly, and marked the whole picture of CSF-CN system in rodent’s brain successfully [25]. We found the dCSF-CNs, with their bodies mainly located in the ventral periaqueductal central gray (PAG) of the brainstem, and their processes penetrate the ependymal cells, and stretched into the CSF, were a distinct group from other neurons. After this anatomical characteristic, we named cerebrospinal fluid-contacting nucleus (CSF-CN) [22,25,30]. Our previous electron microscope observation showed that there were both excitatory and inhibitory synapses between non-CSF-CNs and CSF-CN and found that the axon terminals of CSF-CN labeled by CB-HRP extended directly into the cavity of 3V [22]. The connection between CSF-CN and CSF is via non-synaptic signal transmission, with the possibility that substances can be absorbed from the CSF as well as substances secreted into the CSF. In addition, there is the possibility that CSF-CN could sense the pressure of CSF as well as changes in composition of various neurotransmitters and cell factors. Furthermore, CSF-CN can transmit the information to other areas of the brain [31].

Transient receptor potential receptors (TRP) on primary afferent neurons respond to noxious and/or thermal stimuli. TRPV1 receptor can be activated by noxious heat, acid, capsaicin and resiniferatoxin,
present in CSF-CN and may mediate dCSF-CNIs participating in a series of pathological and physiological activities. This finding provides a new starting point to study functions of CSF-CN more deeply.

A rapidly growing body of evidence suggests a functional role for the TRPV1 channel family in brain function [17,26-29]. The CSF-CN connects the CSF and the brain parenchyma, and have a more important function than the subependymal and ependymal CSF-CNIs. Our previous studies have demonstrated that the dCSF-CNIs are closely linked to inflammatory and neuropathic pain, and morphine dependency and withdrawal [23,30]. However, no previous studies have focused on the relationship between CSF-CN and TRPV1 and pain sensation. Therefore, we speculate that the dCSF-CNIs participate in pain modulation via the receptor TRPV1. Our immunohistochemical analysis showed the TRPV1 expression levers were significantly increased in the CSF-CN at 10 days after CCI surgery. The up-regulation of the TRPV1 at central nervous system provides morphological evidence that the sensitivity of the vanilloid system is increased in neuropathic pain conditions, therefore making the TRPV1 channel a potential candidate for the future development of novel neuropathic pain relieving agents.

Furthermore, we report that lateral ventricle (LV) administration of SB366791 (VR1 specificity antagonist) can produce antihyperalgesic in rats with neuropathic pain, in other words, SB366791 reduced already established mechanical allodynia and thermal hyperalgesia 10 days after CCI. These praxiological dates are consistent with the morphological evidence that TRPV1 receptor is up-regulated at the CSF-CN in neuropathic pain condition. The existence of TRPV1 immunoreactivity and analgesia effect induced by an intracerebroventricular (i.c.v.) injection of VR1 antagonist, suggest that the CSF-CN transmits thermal and pain sensation to non-CSF-CNIs in the central nervous system neurons via TRPV1 in neuropathic pain.

The mechanism of neuropathic pain is complicated. Previous studies have shown that peripheral nerve injury not only caused the change of synaptic connections in spinal cord, but also caused the change in supraspinal central [35]. There was evidence that TRPV1 receptors were necessary and sufficient for a form of long-term depression at excitatory synapses. TRPV1 mediated LTD may have a role in long-term change in physiological and pathological circuit behavior [36]. The broad distribution of TRPV1 receptors in the brain suggests that these receptors could play a similar role in synaptic plasticity throughout the CNS [37,38]. We speculated that the underline mechanism may be that up-regulation of TRPV1 in CSF-CN altered function and structure of the CSF-CN causing the development of neuropathic pain, which participated in the mechanical allodynia and the thermal hyperalgesia in CCI rats.

TRPV1 receptor blockers were proven to be analgesics in neuropathic pain conditions in PNS. TRPV1 receptors have been identified in various regions of the brain known for their role in pain transmission or modulation [14-16] such as amygdale, solitary tract nucleus, somatosensory cortex, anterior cingulated cortex and insula [39,40]. VR1 is also expressed in periaqueductal grey (PAG) and rostral ventromedial medulla (RVM) whose activation leads to analgesia [41]. Such an effect is associated with a glutamate increase and the activation of OFF and inhibition of ON cell population in the RVM. Activation of the antinociceptive descending pathway via TRPV1 receptor simulation in the PAG may be a novel strategy for producing analgesia in chronic pain [41]. Conversely, according to the relationship between CSF-CN and ventricular, our data suggest that the blockade of TRPV1 receptors in the CSF-CN could contribute to the analgesia effect in neuropathic pain. Unlike other nucleus, our studies are well consistent with the role of supraspinal TRPV1 receptor on pain transmission and modulation which was evidenced by intracerebroventricular (i.c.v.) SB366791, capsazepine or ruthenium red, another TRPV1 receptor antagonist injections which attenuated nocifensive behavior induced by an intradermal injection of capsaicin or formalin in mice [42]. Relatively, i.c.v. injection of capsaicin, decreased nociceptive threshold and reduced morphine and stress-induced analgesia [43,44,45]. In addition, labeling the CNS using CB-HRP is poor, but it is an ideal dCSF-CN tracer which is absorbed by neurons via their receptor [22]. Based on our prior experiment, we assumed that the CSF-CN is different from the general central nervous neurons and their function is similar to that of peripheral neurons. Similar to peripheral sites, sensitization associated with an up-regulation of CSF-CN TRPV1 receptor is thought to be at the base of the development of mechanical allodynia and thermal hyperalgesia in the chronic constriction injury of the sciatic nerve.

Recently, there has been great interest in therapeutic agents targeting TRPV1 receptors for several disorders. There is also evidence that TRPV1 receptors in the CNS are involved in pain modulation and might serve as useful drug targets. Our results as well as others indicate that bind to CSF-CN TRPV1 receptors could provide novel drug targets for neuropathic pain. These data presents a valid strategy and for pain relief and open up the perspective of curing currently untreatable conditions- neuropathic pain.

Conclusion

Our data suggest that the TRPV1 is expressed in the CSF-CN. The expression of TRPV1 was increased in CSF-CN by CCI surgery. Furthermore, i.c.v. administration of SB366791 exhibited an anti-allodynic effect in CCI rats. The CSF-CN may play the important roles in neumo modification and neuroendocrine regulation between brain parenchyma and CSF.

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