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Possible Involvement of Brain-Derived Neurotrophic Factor in Analgesic Effects of 4-Methylcatechol on Neuropathic Pain

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Summary Excessive neurotransmission related to derangements of intracellular signaling of the spinal and bulb-spinal systems is a possible mechanism underlying neuropathic pain. It is suggested that lack of brain-derived neurotrophic factor (BDNF) may result in these derangements. BDNF is induced in cultured neurons by 4-methylcatechol (4-MC), but the role of the 4-MC-induced BDNF in neuropathic pain is poorly understood. The present study examined the effects of 4-MC on expression of pERK and c-FOS in relation to neuropathic pain. A chronic constriction injury (CCI) model was prepared in rats and pain was assessed based on paw withdrawal latency (PWL) to heat stimuli. 4-MC was injected after induction of CCI. The expression of c-FOS and pERK was assessed by counting the number of immunopositive cells. On day 14 after CCI, 4-MC reduced persistent decrease in PWL after CCI and this effect was reversed by K252a (Trk-B receptor inhibitor) and SP600125 (JNK-1 inhibitor), but not by Minocyclin (microglia inactivator) or SB203580 (p38-MAPK inhibitor). In addition, 4-MC attenuated CCI-induced increases in pERK and c-FOS immunoreactivities. These results suggest that 4-MC ameliorates chronic pain by preventing abnormal BDNF/Trk-B signaling.

Key words: chronic pain, BDNF, 4-methylcathecol, JNK-1, pERK

Introduction

Neuropathic pain following peripheral nerve injury or spinal cord injury is a significant clinical concern. This pain may reflect excessive neurotransmission related to abnormal intracellular signaling, and dysfunction of interneuron, and glia cell activation in the spinal cord.[1)2)3)] Accordingly, analgesic effects may occur through reduction of the enhancement of spinal neurotransmission mediated by intracellular signaling and glia cells.[2)4)5)] Mitogen-activated protein kinases play a key role in intracellular signaling via post-translational, translational and transcriptional regulation. Under conditions of neuronal damage, extracellular signal-regulated kinase (ERK) is activated in spinal cord neurons and glia in the central nervous system (CNS).[6)] Intracellular pERK activity is thought to be the most important for signaling after central sensitization is evoked physically or pharmacologically.[7)] c-FOS, an immediate early gene, is important markers for neuronal activations. Opioid or N-methyl D-aspartate (NMDA) receptor antagonists suppress spinal c-FOS expression evoked by nociception.[8)] This suggests that receptor-operated activation may be involved in c-FOS induction in the spinal cord. Thus, excessive neurotransmission, which can be reflected by increases in pERK and c-FOS expression, may be a pivotal mechanism in neuropathic pain.

Neurotrophic factors including brain-de-

rived neurotrophic factor (BDNF) play important roles in neuronal growth, differentiation, survival, and recovery from neuronal damage, and also work as central modulators of emotional function and pain transmission.[9] Spinally transplanted cells containing BDNF can attenuate allodynia and hyperalgesia in rat sciatic nerve injury.[10] Thus, spinal BDNF may influence chronic pain through an activation of synaptic neurons via the Trk-B receptor at pre-and post-synaptic neurons. Catecholamines increase the nerve growth factor (NGF) level in mouse astrocytes and 4-methylcatechol (4-MC), an alkylcatechol, also induces neurotrophic factors in rodent astrocytes.[11] Repetitive administration of 4-MC can lead to an increase of BDNF in cultured rat brains and in vivo in CNS[12] with reversal of derangement in neuropathies.[13][14] Thus, 4-MC may contribute to recovery from neuropathic pain by modulating the induction of BDNF, as suggested in cultured neurons and astrocytes, but the specific mechanism is not understood. These backgrounds suggest that 4-MC may prevent derangement of pain-related neurotransmission by inducing BDNF and normalizing cell responses, as reflected by pERK activity.

Thus, we aimed to examine the analgesic effects of 4-MC on rat neuropathic pain and involvement of BDNF signaling including Trk-B receptors, p38-MAPK and JNK-1 in the 4-MC-induced analgesia. We also examined if glia activation was involved in the analgesic effects of 4-MC.

Materials and methods

This study was performed with approval from the Institutional Animal Care and Use Committee and followed the Yamaguchi University Graduate School of Medicine Guidelines for the Care and Use of Laboratory Animals.

Animals and surgical operations

Sprague-Dawley rats (Kyudo Co., Saga, Japan) weighing 250-350g were used in the study. A PE-10 intrathecal catheter was inserted in rats scheduled for spinal drug treatment.[15] Briefly, under 3% halothane anesthesia with O_2, the atlanto-occipital membrane was exposed and an intrathecal PE-10 8.5 cm catheter was inserted caudally through the subarachnoid space to the lumbar area. The rats were left to recover for 3 days after catheter insertion. The neuropathic pain model was made using a specific chronic constriction injury (CCI) of the left sciatic nerve.[16] Under sodium pentobarbital anesthesia (30mg/kg, i.p.), the common sciatic nerve was exposed and four loose ligatures of 4-0 silk thread were tied around the nerve proximal to the trifurcation at a distance of 1 mm.

Pain behavior test

Thermal hyperalgesia was based on paw withdrawal latency (PWL) using Hargreaves' plantar test apparatus (No. 7370, Ugo Basile, Italy). The rat was placed on a Plexiglas floor and acclimatized for 15 min. PWL in both hind paws was measured five times alternately with an interval of at least 5 min and averaged. Measurements were made before and 3, 7, 10 and 14 days after CCI. PWL was calculated by subtraction of the latency of the contralateral side from that of the injured side.

Immunohistochemistry

On day 14 after CCI, the animals were anesthetized with 3% halothane and perfused with saline followed by 4% paraformaldehyde through the right atrium. Sections of the lumbar enlargement (L_{3-5}) were prepared at 10-μm thickness and subjected to immunohistochemistry. The avidin-biotin complex (ABC) method was used to assess the number of c-FOS- and pERK-positive cells using rabbit anti c-FOS antibody (×5000, polyclonal, Code No.PC38, Calbiochem, Denmark), and rabbit anti-pERK1/2 antibody (×200, polyclonal, Code No.9101, Cell Signaling Technology, Beverly, MA,USA), respectively. After staining (DAB (Lot.CM236, Dojindo, Japan)), immunopositive cells in the pain pathway and other regions related to emotional function of the CNS on each side were counted at 200× to 400× magnifications. Histochemical assessments were performed in a double-blind manner and the total numbers of cells counted in three neighboring sections were averaged.

Study protocol

Rats were randomly divided into eight groups, as defined below. 4-MC (1μg/kg, Sigma, No. M34200, Tokyo, Japan) was injected i.p. daily

from day 7 until day 14 after CCI in groups (3) to (8). For manipulation of the analgesic effects of 4-MC, all drugs were injected intrathecally (i.t.). PWL was measured 4 hours after administration of 4-MC. The eight groups were as follows:

(1) Sham (n=15): anesthesia and skin incision only and observation for 14 days.
(2) Untreated: UT (n=12): saline (0.5 ml, Otsuka Pharmacy, Tokushima, Japan) injected i.p. daily from day 7 after CCI.
(3) 4-MC + Saline (n=12): saline i.t. and 4-MC i.p. daily from day 7 until day 14 after CCI.
(4) 4-MC + Minocyclin (n=6): Minocyclin (60μg/10μl; M9511, Sigma-Aldrich, Tokyo, Japan), a microglia inactivator, was injected i.t. on day 14.
(5) 4-MC + SB (n=6): SB203580 (5μg/10μl; Calbiochem, Denmark), a p38-MAPK inhibitor, was injected i.t. on day 14.
(6) 4-MC + SP (n=6): SP600125 (25μg/10μl; Calbiochem, Germany), a JNK-1 inhibitor, was injected i.t. on day 14.
(7) 4-MC + anti-BDNF (n=3): anti-BDNF antibody (AF248, R&D Systems, Minneapolis, USA) was injected i.t. on day 14.
(8) 4-MC + K252a (n=6): K252a (25μg/10μl; LKT Laboratories, Inc, USA), a Trk-B receptor inhibitor, was injected i.t. on day 14.

Statistical analysis

Data are expressed as means ± standard error of means (SEM). Statistical analysis was performed using two-way ANOVA followed by a Tukey PSLD test for multiple comparisons of PWL and cell count among groups. $P < 0.05$ was considered to be statistically significant.

Results

Effect of 4-MC on PWL

Rats given saline i.p. without CCI (Sham group) showed no changes in PWL over the two-week experimental period. In the Untreated group, rats showed a significant decrease in PWL on postoperative day 7 and day 14 after CCI . The decrease in PWL on day 14 was significantly reduced in 4-MC-treated animals compared with the Untreated group (Fig. 1).

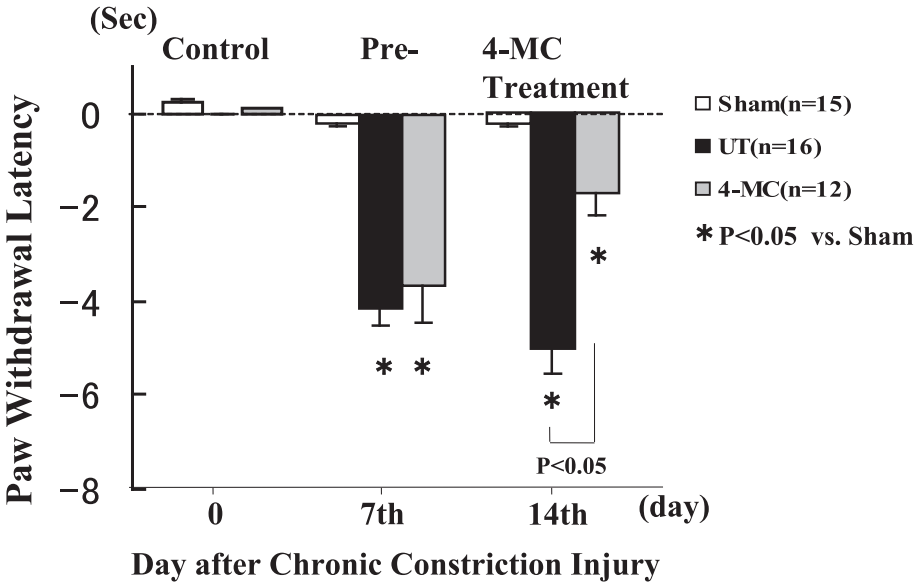

Fig. 1 The effect of 4-methylcatechol (4-MC) on paw withdrawal latency (PWL, sec) after chronic constriction injury (CCI). The chronic administration of 4-MC (1μg/kg, i.p.) restored a decrease in PWL in response to thermal stimuli of an injured hind paw. UT:Untreated. Values are shown as the mean ± SEM. *: $P < 0.05$ vs. Sham.

Modification of analgesic effects of 4-MC by intracellular signaling

The decrease in PWL observed in the Untreated group was significantly reduced on day 14 in 4-MC-treated rats. The 4-MC induced reduction in the decrease in PWL was significantly reversed by K252a and SP600125, but not by Minocyclin and SB203580 (Fig. 2).

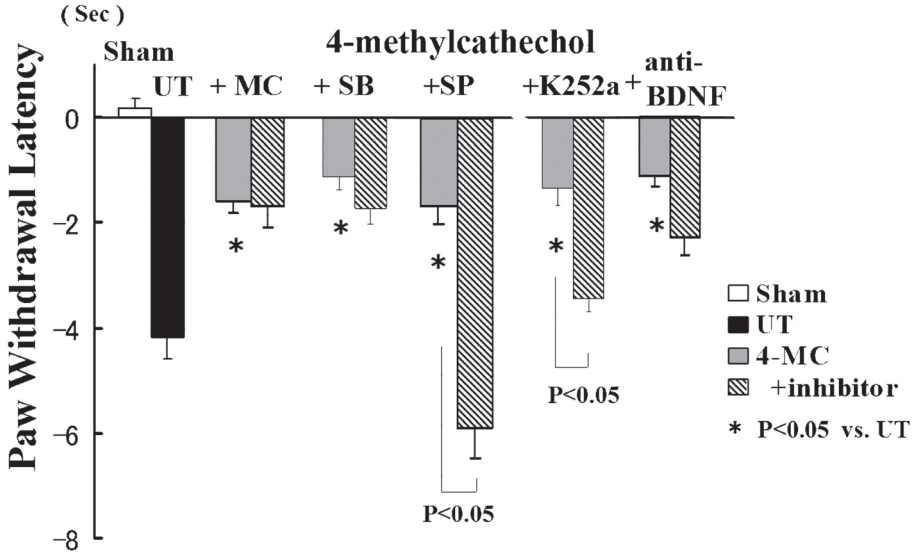

Fig. 2 Modulation of the analgesic effect of 4-methylcatechol (4-MC) by drugs associated with intracellular signaling. The analgesic effects of 4-MC was reversed by a Trk-B receptor antagonist, K252a and a JNK-1 inhibitor, SP600125 (SP), but not by a microglia inactivator, Minocyclin (MC) and p38-MAPK inhibitor, SB203580 (SB). UT:Untreated. Values are shown as the mean ± SEM. *: P<0.05 vs. Untreated.

Effects of 4-MC on c-FOS and pERK immunoreactivities in the spinal cord and brain

Representative microphotographs for c-FOS and pERK expression are shown in Figs. 3A and 3B, respectively. On day 14 after CCI, the numbers of c-FOS- and pERK-immunopositive cells evoked by persistent pain increased in the regions related to ascending pain pathways (dorsal horn of the spinal cord, thalamus VPL/VPM, somato-sensory cortex S1), as well as in the regions related to emotional function (anterior cingulate cortex, amygdala, and hypothalamus). These increases in pERK and c-FOS expression were significantly attenuated by 4-MC treatment (Table 1) . A schematic drawing of the distribution of c-FOS and pERK-immunopositive cells is shown in Fig. 4.

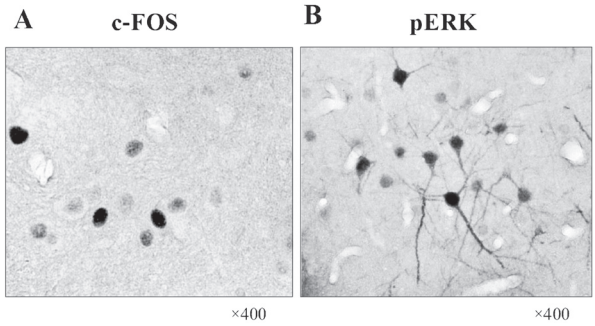

Fig. 3 Representative microphotographs for c-FOS (A) and pERK (B) immunoreactivities, which are in the periaqueductal gray (A) and anterior cingulate cortex (B) on day 14 following sciatic nerve ligation (Untreated).

Table 1 Regional change in c-FOS and pERK immunoreactivities following peripheral nerve injury

Structures	c-FOS			pERK		
	Sham	Untreated	4-MC	Sham	Untreated	4-MC
Spinal Cord(Rexed I-II)						
Thoracic Ipsilateral	0±0.0	1±1.3	0±0.0	0±0.0	0±0.0	0±0.0
Thoracic Contralateral	0±0.0	2±1.7	0±0.0	0±0.0	0±0.0	0±0.0
Lumbar Ipsilateral	0±0.0	10±3.8 *	2±0.8 #	0±0.0	8±0.6 *	3±1.2 #
Lumbar Contralateral	0±0.0	3±0.3	1±0.3	0±0.0	1±1.0	1±0.6
Midbrain assending						
Periaqueductal Gray	8±2.7	28±9.8 *	11±2.7 #	9±7.8	19±3.2 *	9±2.7 #
Relayed neurons						
Thalamus VPL,VPM	0±0.0	10±1.8 *	5±2.0 #	0±0.0	5±2.3	3±0.6
3rd order						
Sensory Cx. S1	3±0.1	36±2.3 *	20±3.6 #	2±1.2	11±3.7 *	2±0.9 #
Limbic systems						
Ant. Cingulate Cx.	7±1.6	33±6.6 *	12±1.2 #	7±4.3	32±6.8 *	7±1.2 #
Ant. Insula	3±2.7	52±4.9 *	25±5.8 #	4±1.6	47±7.5 *	28±5.8 #
Hippocampus CA1-4, DG	2±0.3	26±5.9 *	13±2.8	2±0.9	13±3.5 *	9±1.2
Amygdala	4±2.2	19±8.1 *	12±3.0	5±3.1	17±4.0 *	10±4.2
Hypothalamus	8±8.3	26±5.2 *	18±6.9	15±6.9	30±9.0 *	13±1.2 #
Descending inhibitory systems						
Dorsal Raphe	2±0.6	4±4.0	4±4.0	1±0.6	5±2.7	1±1.0
Locus Coeruleus	0±0.0	0±0.0	0±0.0	0±0.0	4±4.0	0±0.0

Values are shown as the mean ± SEM.
*:$p < 0.05$ vs. Sham. #:$p < 0.05$ vs. Untreated.

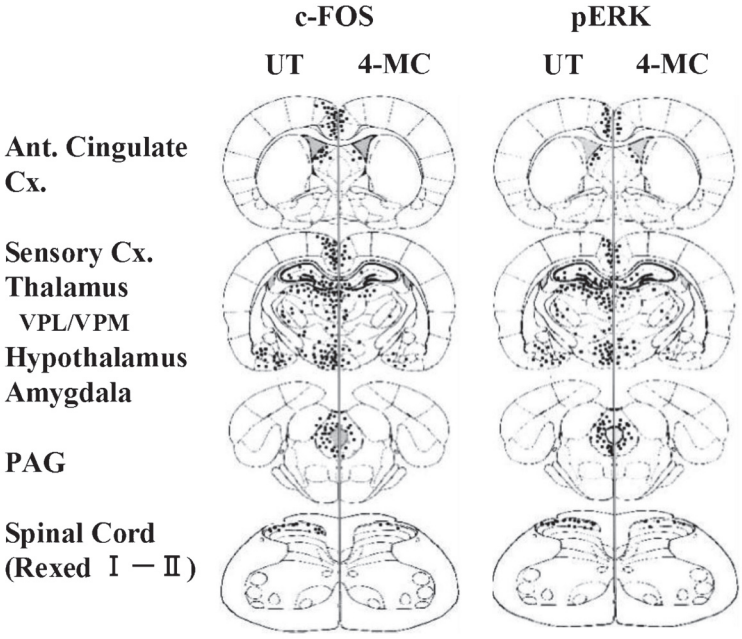

Fig. 4 Schematic illustration of c-FOS and pERK immunoreactivities in the central nervous system. (Left : UT: Untreated rats, Right : 4-MC treatments)

Discussion

The CCI model used in this study involves mild sciatic nerve injury that is pathologically and physiologically applicable to clinical situations of slow development of chronic pain. Many studies have proposed dis-inhibition mechanisms based on neuronal degeneration of regulatory interneurons of the spinal cord,[3)10)] suggesting a lack of neurotrophic factors during the chronic pain state.

BDNF that is localized widely in the sensory and limbic systems is essential for the maintenance of memory, emotional regulation (depression),[9)] and persistent pain.[18)] A recent study showed that BDNF is a potent modulator of neurotransmission through activation of Trk-B receptors followed by intracellular phospholilation, including pERK activity.[6)] Thus, a lack of BDNF may be a pivotal mechanism underlying chronic pain.

We found that 4-MC significantly improved chronic pain after CCI, suggesting that lack of BDNF in the CNS may result in chronic pain. Fukumitsu, et al.[12)] showed that a dose of 4-MC (2μg, i.p.) similar to that used in the present study accelerated the synthesis of BDNF in the CNS for 32-40 hours. Thus, sufficient BDNF levels were thought to be present in the CNS in the chronic stage in the current study, since 4-MC was injected daily for 7 days.

Our results showed that CCI-evoked thermal hyperalgesia was attenuated by 4-MC, and this effect was reversed by a Trk-B antagonist, K252a, and by a JNK-1 antagonist, SP600125, but not by a microglia inactivator, Minocyclin or microglia related p38-MAPK inhibitor, SB203580. These results are in good agreement with a report showing that BDNF injection prevents hypersensitivity following noxious stimuli.[10)] In fact, we found that 4-MC normalized pERK activity in the spinal and supraspinal cord. This suggests that 4-MC induces BDNF that may prevent damage of neurons and glia cells via Trk-B signaling pathway that is related to JNK-1 as well as pERK and c-FOS. These findings are supported by evidence that 4-MC stimulates BDNF synthesis in cultured infant rat brains[11)] and can induce BDNF mRNA.[12)]

pERK is activated by neural excitation, and there is considerable evidence showing involvement of ERK in pain hypersensitivity.[19)] The molecular mechanisms underlying ERK-mediated pain hypersensitivity have been examined,[19)] but findings in descrete brain regions related to pain and emotion have not been examined previously. Our results showed that pERK activation occurred in regions similar to those activated by chronic pain, as observed by fMRI in humans, and demonstrated that the activated regions are related to sensory systems (spinal cord, thalamus VPL/VPM, sensory cortex, PAG, raphe dorsalis) and emotional systems (insular, anterior cingulate cortex, amygdala) in chronic pain.[20)]

After peripheral tissue and nerve injury, c-FOS begins to be induced after 30-60 minutes, whereas the pERK level correlates well with the development of pain hypersensitivity. While c-FOS is often induced in the nuclei of neurons, pERK can be induced in different subcellular structures of neurons, including nuclei, cytoplasm, axons, and dendrites. Therefore, c-FOS and pERK levels both could reflect neuronal activation during chronic pain. In the present study, we found that 4-MC ameliorates chronic pain possibly by preventing activation of pERK in various structures related to the pain pathway and pain-emotion (limbic) systems as shown in Table 1 and Fig. 4. This is in good agreement with the finding by Zhuang et al.[19)] that spinal nerve ligation induced sequential activation of pERK in the spinal cord. Our results clearly show that 4-MC has beneficial effects on chronic pain. Thus, the therapeutic value of 4-MC for neuropathic pain in humans warrants further investigation.

In summary, we suggest that lack of BDNF and pERK activation may be involved in the induction of chronic pain. 4-MC attenuates chronic pain and the analgesic effects of 4-MC is reversed by inhibitors of JNK-1 and Trk-B. Therefore, 4-MC may exert the analgesic effects on chronic pain via BDNF/Trk-B signaling pathway.

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