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RESEARCH PAPER

Increase in hemokinin-1 mRNA in the spinal cord during the early phase of a neuropathic pain state

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Background and purpose: Substance P (SP), a representative member of the tachykinin family, is involved in nociception under physiological and pathological conditions. Recently, hemokinin-1 (HK-1) was identified as a new member of this family. Although HK-1 acts on NK_1 tachykinin receptors that are thought to be innate for SP, the roles of HK-1 in neuropathic pain are still unknown.
Experimental approach: Using rats that had been subjected to chronic constrictive injury (CCI) of the sciatic nerve as a neuropathic pain model, we examined the changes in expression of SP- and HK-1-encoding genes (TAC1 and TAC4, respectively) in the L4/L5 spinal cord and L4/L5 dorsal root ganglia (DRGs) in association with changes in pain-related behaviours in this neuropathic pain state.
Key results: The TAC4 mRNA level was increased on the ipsilateral side of the dorsal spinal cord, but not in DRGs, at day 3 after CCI. In contrast, the TAC1 mRNA level was significantly increased in the DRGs at day 3 after CCI without any changes in the dorsal spinal cord. Analysis of a cultured microglial cell line revealed the presence of TAC4 mRNA in microglial cells. Minocycline, an inhibitor of microglial activation, blocked the increased expression of TAC4 mRNA after CCI and inhibited the associated pain-related behaviours and microglial activation in the spinal cord.
Conclusions and implications: The present results suggest that HK-1 expression is increased at least partly in activated microglial cells after nerve injury and is clearly involved in the early phase of neuropathic pain.
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Keywords: chronic constrictive injury; dorsal root ganglion; hemokinin-1; neuropathic pain; microglial cell; spinal cord; substance P; TAC1; TAC4; tachykinin

Abbreviations: CCI, chronic constrictive injury; DRG, dorsal root ganglion; HK-1, hemokinin-1; PNS, peripheral nervous system; SP, substance P

Introduction

Mammalian tachykinins comprise a family of peptides with a common carboxyl terminal amide motif (Patacchini *et al.*, 2004). Substance P (SP), a representative member of this family, is mainly distributed in the peripheral nervous system (PNS) and CNS and serves as a neurotransmitter in higher brain functions such as emotion and pain perception (Otsuka and Yoshioka, 1993; Severini *et al.*, 2002). SP is expressed in nociceptive dorsal root ganglion (DRG) neurons with small cell bodies, and is released upon stimulation. The released SP activates its innate receptor NK_1 on second-order neurons in the spinal cord and transmits intense noxious signals to the higher CNS (Hill, 2000; Mantyh and Hunt, 2004). Studies using knockout mice and selective NK_1 receptor antagonists have revealed that the SP-NK_1 system contributes to a variety of pathological pain states, including inflammatory and neuropathic pain (Hill, 2000; Mantyh and Hunt, 2004). In neuropathic pain, which is characterized by severe chronic pain caused by damage to the PNS or CNS (Scholz and Woolf, 2007), SP and NK_1 play a crucial role in spinal neuron sensitization through modulation of NMDA receptor gating (Woolf and Salter, 2000).

Recently, hemokinin-1 (HK-1) has been identified as a novel tachykinin peptide involved in the development of the B cell lineage (Zhang *et al.*, 2000). HK-1 binds to NK_1 receptors with high affinity, similar to SP (Kurtz *et al.*, 2002; Page, 2004). The TAC1 gene encoding SP and neurokinin A is mainly expressed in the nervous systems, whereas the TAC4 gene-encoding HK-1 is expressed in various types of tissues

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and cells, including immune cells (Patacchini *et al*., 2004). Although HK-1 and SP have been shown to cause similar, but distinct, pain-related behaviours (Endo *et al*., 2006), no studies have examined how HK-1 behaves during inflammatory or neuropathic pain. Thus, the roles of HK-1 in physiological or pathological pain states, such as neuropathic pain, are still unknown.

Microglial cells are considered to be CNS-resident macrophages and are present in all regions of the CNS where they constantly screen its environment (Hanisch and Kettenmann, 2007). Upon peripheral nerve injury, microglial cells in the spinal cord transform from their resting phenotype to their activated phenotype (Tsuda *et al*., 2005) and secrete a variety of molecules, thereby contributing to the development of neuropathic pain (Tsuda *et al*., 2005; Hanisch and Kettenmann, 2007). As HK-1 is expressed in microglial cells (Nelson *et al*., 2004), it may play a role in neuropathic pain through activated microglial cells.

In the present study, we examined TAC4 mRNA expression in a rat model of neuropathic pain and compared it with TAC1 mRNA expression so as to clarify the specific contribution of HK-1 to neuropathic pain.

Materials and methods

Animals

All experimental procedures were approved by the Nippon Medical School Animal Care and Use Committee (approval number H19-053) and carried out in accordance with the guidelines of the International Association for the Study of Pain (Covino *et al*., 1980). Six-week-old male Sprague–Dawley rats, weighing 150–170 g at the time of surgery, were used for all experiments. The rats were housed singly in an animal house for at least 5 days before the experiments, and water and food were available *ad libitum*.

Chronic constrictive injury (CCI) was basically produced as described in a previous report (Bennett and Xie, 1988), except that 4−0 silk thread was used instead of chromic gut. Briefly, rats were deeply anaesthetized with sodium pentobarbital ($50\,mg\,kg^{-1}$, i.p.). The left (ipsilateral) sciatic nerve was exposed at the mid-thigh level, and four 4−0 silk threads were loosely ligated around the nerve at intervals of approximately 1 mm. The incision was closed with a 4−0 silk suture. The right (contralateral) sciatic nerve was left intact. In sham-operated rats, the operation was performed in the same manner except that sciatic nerve ligation was not carried out. Naive control rats were housed without an operation until tissue sampling. After 1, 3 and 7 days, the animals were deeply anaesthetized and the portions of the ipsilateral and contralateral dorsal quadrants of the L4/L5 spinal cord and the L4/L5 DRGs were immediately dissected. Each sample was stored at −80 °C until use for RNA extraction.

Minocycline administration

Minocycline hydrochloride, an inhibitor of microglial activation (Yrjänheikki *et al*., 1998), was dissolved in sterile saline and administered to CCI rats at a dose of $90\,mg\,kg^{-1}$ (i.p.) immediately after the CCI operation and then at $45\,mg\,kg^{-1}$ every 12 h for 3 consecutive days according to a previous study (Yune *et al*., 2007). Saline alone was administered to CCI rats as a control. The rats were killed for mRNA quantification and immunohistochemistry immediately after the behavioural tests on day 3.

Behavioural tests

Mechanical allodynia and thermal hyperalgesia were examined on day 0 before the surgery and at days 1, 3 and 7 after CCI as previously described (Nagano *et al*., 2003). Paw withdrawal in response to mechanical stimuli was measured using a set of von Frey filaments with bending forces ranging from 1.0 to 52.8 g. Each rat was placed on a metallic mesh floor covered with a plastic box and a von Frey monofilament was applied to the plantar surface of the contralateral or ipsilateral hind paw from underneath the mesh floor. Each paw was stimulated with each filament five times in an individual trial. The weakest force (g) inducing withdrawal of the stimulated paw at least three times in each trial was referred to as the paw withdrawal threshold. The Plantar Test (Ugo Basile, Varese, Italy) was used to examine thermal hyperalgesia. Each rat was placed on a glass plate with a radiant heat generator underneath. After an acclimatization period, radiation heat was independently applied to the pad of the contralateral or ipsilateral hind paw. The latency of paw withdrawal from the heat stimuli was measured three times at 5-min intervals, and the average value was used as the latency of the response.

Real-time PCR

Quantitative analyses of rat TAC1 and TAC4 mRNA levels were performed as previously described (Nagano *et al*., 2006). Total RNA was extracted from the L4/L5 dorsal spinal cord and L4/L5 DRGs using RNAiso and purified using an RNeasy Mini Kit according to the manufacturers' instructions. To avoid contamination by genomic DNA, the extracted RNA was treated with 10 μL of DNase ($0.125\,u\,\mu L^{-1}$) at 37 °C for 30 min while on the column of the RNeasy Mini Kit. First-strand cDNAs were synthesized using 0.5 μg of total RNA in each tube with Oligo (dT) primer, dNTP mix and Superscript II Reverse Transcriptase. For PCR amplification, 1 μL of the first-strand cDNAs was added to 19 μL of a reaction mixture containing TaqMan Gene Expression Master Mix, 900 nM of each primer and 250 nM of TaqMan Probe and amplified using a GeneAmp 5700 sequence detection system. PCR primers specific for rat TAC1 and TAC4 were designed based on the cDNA sequences deposited in GenBank (NM_012666 and AY471575, respectively) using Primer Express version 2.0. The forward primers, reverse primers and probes were 5′-CGCAATGCAGAACTACGAAAGA-3′, 5′-CGCGGACACAGATGGAGAT-3′ and 5′-CGTAAATAAACCCTGTAACGCACTATCTAT-3′ for TAC1 and 5-AGGGCTCGATAAAGGAGTTA-3′, 5-TTCAGCCCTCTACCCAGCAT-3′ and 5′-TAGGCAGCTTCCTCAGC-3′ for TAC4, respectively. For quantification, the cDNA sequences of TAC1 and TAC4 were inserted into the pGEM-T easy vector and pBluescript II SK (+) vector, respectively. The plasmids were then serially diluted to concentrations of 1.0×10^1–1.0×10^6 mol per reaction tube for use as standards. All PCRs using the standards and samples were

performed in triplicate at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The number of cDNA copies was calculated using a standard curve obtained from the set of control plasmids in each assay.

Immunohistochemistry

The animals were deeply anaesthetized with pentobarbital (i.p.) and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) followed by freshly prepared 4% paraformaldehyde in PBS. The L4 spinal cord was dissected out, post-fixed in the same fixative at 4 °C overnight and then cryoprotected in 20% sucrose in PBS at 4 °C overnight. Subsequently, the spinal cord was rapidly frozen in dry ice/acetone and cut into 10-μm transverse sections using a cryostat. The sections were preincubated in PBS containing 5% normal donkey serum and 0.2% Triton X-100 at room temperature for 30 min, and then incubated with a mouse monoclonal anti-CD11b antibody (OX-42, a microglial activation marker (Ling *et al*., 1990); 1:1000 dilution) at 4 °C overnight. After being washed in PBS, the sections were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:1000 dilution). Images were captured using a high-resolution digital camera equipped with a computer (Olympus, Tokyo, Japan).

Microglial cell culture

An immortalized rat microglial cell line, GMI-R1, was originally established from a neonatal Fisher 344 rat brain primary culture using a non-enzymatic and non-virus-transformed procedure (Salimi *et al*., 2002). These microglial cells were maintained in Eagle's minimal essential medium (M4655), supplemented with 0.2% glucose, 5 μg mL^{-1} insulin (I5500) and 10% foetal calf serum at 37 °C under a 5% CO_2/95% air atmosphere. Mouse recombinant granulocyte-macrophage colony-stimulating factor (1 ng mL^{-1}) was added as a supplement in the culture medium to maintain the cells in a proliferative state. The culture media were renewed two times per week. For mRNA expression analyses, the cells were harvested by scraping in a lysis buffer (RLT buffer in the RNeasy Mini Kit) and stored at −80 °C until use. First-strand cDNAs were prepared as described above for the real-time PCR procedure. PCR was performed at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min using forward and reverse primer pairs for TAC4 (forward, 5-AGGGCTCGATAAAGGAGTTA-3′; reverse, 5-TTCAGCCCTCTACCCAGCAT-3′; 62-bp product) or NK_1 (NM_012667; forward, 5′-CACCCGATACCTCCAGAC ACA-3′; reverse, 5′-GGAGCCGTTGGAGGTGAGA-3′; 148-bp product) and 25 mu μL^{-1} Ampli*Taq* Gold DNA polymerase. The amplified PCR products were electrophoresed in a 2% (w v^{-1}) agarose gel and stained with ethidium bromide. An image of the agarose gel was captured using a BioDoc-It System UV Transilluminator.

Materials

Minocycline hydrochloride was obtained from Sigma-Aldrich (St Louis, MO, USA); von Frey filaments, Muromachi Kikai (Tokyo, Japan); RNAiso, Takara (Shiga, Japan) RNeasy Mini Kit (Qiagen, Valencia, CA, USA); Dnase, Promega (Madison, WI, USA). Oligo (dT) primer, dNTP mix and Superscript II Reverse Transcriptase were from Invitrogen (San Diego, CA, USA). The TaqMan Gene Expression Master Mix, TaqMan Probe, GeneAmp 5700 sequence detection system and Primer Express version 2.0 were from Applied Biosystems (Foster City, CA, USA); pGEM-T easy vector, Promega and pBluescript II SK (+) vector, Stratagene (La Jolla, CA, USA). The cryostat was from Leica (Tokyo, Japan); mouse monoclonal anti-CD11b antibody, Serotec (Cambridge, UK); Alexa Fluor 488-conjugated anti-mouse secondary antibody, Invitrogen. Eagle's minimal essential medium and insulin, Sigma-Aldrich; mouse recombinant granulocyte-macrophage colony stimulating factor, Genzyme (Cambridge, MA, USA); Ampli*Taq* Gold DNA polymerase, Applied Biosystems. The BioDoc-It System UV Transilluminator was obtained from BM Equipment (Tokyo, Japan).

Statistical analysis

Values are expressed as means ± s.e.mean. In behavioural tests, differences in the threshold or latency values before and after surgery were analysed by one-way ANOVA, followed by individual *post hoc* multiple comparisons (Dunnett's test) and the paired *t*-test was used to compare these values between the hind paws on the ipsilateral and contralateral sides of each rat. In real-time PCR experiments, differences in the amounts of the products before and after surgery were analysed by one-way ANOVA, followed by individual *post hoc* multiple comparisons (Dunnett's test). For comparison of the amounts of PCR products between the ipsilateral and contralateral sides of minocycline- and saline-treated rats, the paired *t*-test was used. Differences in the amounts of the PCR products of the ipsilateral side between the minocycline- and saline-treated rats were analysed by the unpaired *t*-test. Values of $P<0.05$ were considered to indicate statistical significance.

Nomenclature

The drug/molecular target nomenclature conforms with BJP's Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Behavioural studies

Before the CCI operation on day 0, the paw withdrawal thresholds of 6-week-old rats in response to stimulation with von Frey filaments were 24.3 ± 0.5 g for the hind paws on both sides ($n=6$; Figure 1a). The latencies of withdrawal from heat stimulation in intact rats were 14.8 ± 2.0 and 14.6 ± 1.4 s for the ipsilateral and contralateral hind paws, respectively ($n=6$; Figure 1b). After the CCI operation, the thresholds of paw withdrawal in response to mechanical and heat stimuli began to decrease significantly on the ipsilateral side, but not the contralateral side, from day 3 and day 1, respectively (Figure 1). The increased sensitivities to both stimuli persisted until at least day 7 after CCI (Figure 1). On day 3 after the sham operation, the paw withdrawal

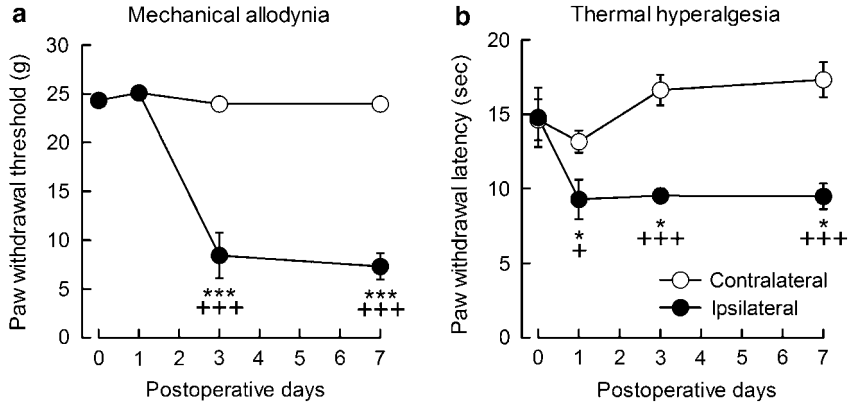

Figure 1 CCI-induced mechanical allodynia (**a**) and thermal hyperalgesia (**b**). The thresholds of paw withdrawal on the ipsilateral side and contralateral side in response to mechanical (**a**) or thermal (**b**) stimuli were measured in rats after CCI. $^{*}P<0.05$ and $^{***}P<0.001$ vs the values on day 0 by one-way ANOVA, followed by Dunnett's *post hoc* test; $^{+}P<0.05$ and $^{+++}P<0.001$ vs the values on the contralateral side at the corresponding days by the paired *t*-test; $n=6$.

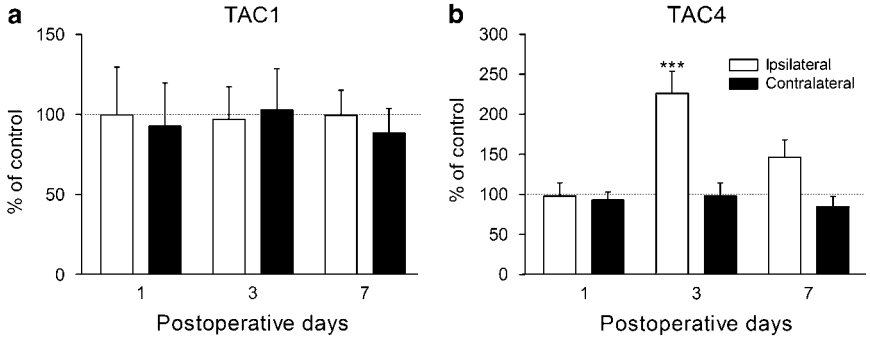

Figure 2 Real-time PCR analysis for TAC1 (**a**) and TAC4 (**b**) mRNA expression levels in the dorsal spinal cord. The levels of mRNA are expressed as percentages of the levels in intact rats. $^{***}P<0.001$ vs the values in intact rats by one-way ANOVA, followed by Dunnett's *post hoc* test; $n=6$.

thresholds were 24.0 ± 0.7 g for the hind paws on both sides ($n=4$), and the latencies of withdrawal from heat stimulation were 21.3 ± 2.0 and 22.3 ± 0.7 s for the ipsilateral and contralateral hind paws, respectively ($n=4$), indicating that the sham operation had no effects on the pain thresholds ($P=0.61$).

TAC1 and TAC4 mRNA expression levels in the dorsal spinal cord following CCI

We examined the expression levels of TAC1 and TAC4 mRNAs in the dorsal spinal cord following CCI. In the intact rats, the numbers of TAC1 and TAC4 mRNA molecules in the dorsal spinal cord were $5.60\times10^{6}\pm9.66\times10^{4}$ and $2.55\times10^{3}\pm4.76\times10^{2}$ mol μg^{-1} total RNA, respectively ($n=6$), indicating that TAC1 mRNA was more abundantly expressed than TAC4 mRNA. After CCI, a significant increase in TAC4 mRNA expression was observed in the dorsal spinal cord on the ipsilateral side at day 3 ($225.96\pm27.92\%$ expressed as a percentage of the value for intact rats; $P<0.001$, vs the value for intact rats; $n=6$; Figure 2b) in association with the development of mechanical allodynia (Figure 1). The increase in TAC4 mRNA expression had almost returned to the baseline level at day 7 after CCI ($146.93\pm21.83\%$; $n=6$; Figure 2b). In contrast, no significant changes were observed for TAC1 mRNA expression on any of the days examined ($n=6$; Figure 2a). In the sham-operated group, the TAC1 and TAC4 mRNA expression levels on the ipsilateral side at day 3 were 110.31 ± 15.48 and $114.32\pm17.81\%$, respectively (expressed as percentages of the values for the contralateral side; $n=4$). There were no significant differences in the TAC1 and TAC4 mRNA expression levels between the two sides of the dorsal spinal cord ($P=0.55$ and $P=0.48$, respectively).

TAC1 and TAC4 mRNA expression levels in DRGs

We examined the expression levels of TAC1 and TAC4 mRNAs in the DRGs. In the intact rats, the numbers of TAC1 and TAC4 mRNA molecules in the L4/L5 DRGs were $6.10\times10^{6}\pm3.97\times10^{5}$ and $2.99\times10^{4}\pm3.47\times10^{3}$ mol μg^{-1} total RNA, respectively ($n=9$). In contrast to the dorsal spinal cord, there were no significant changes in TAC4 mRNA expression during the first 7 days after CCI (Figure 3b). In contrast, the TAC1 mRNA expression level was significantly increased at day 3 after CCI ($121.50\pm3.56\%$ expressed as a percentage of the value for intact rats; $P<0.05$, vs the value for intact rats; $n=9$; Figure 3a). The increase in TAC1 mRNA expression in the DRGs had declined to the baseline level at day 7 after CCI (Figure 3a). The sham operation did not affect the TAC1 and TAC4 mRNA

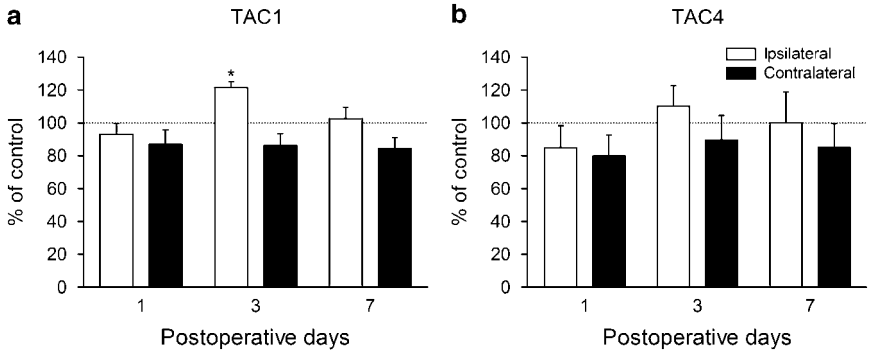

Figure 3 Real-time PCR analysis for TAC1 (**a**) and TAC4 (**b**) mRNA expression levels in the DRGs. The levels of mRNA are expressed as percentages of the levels in intact rats. *$P<0.05$ vs the values in intact rats by one-way ANOVA, followed by Dunnett's *post hoc* test; $n=9$.

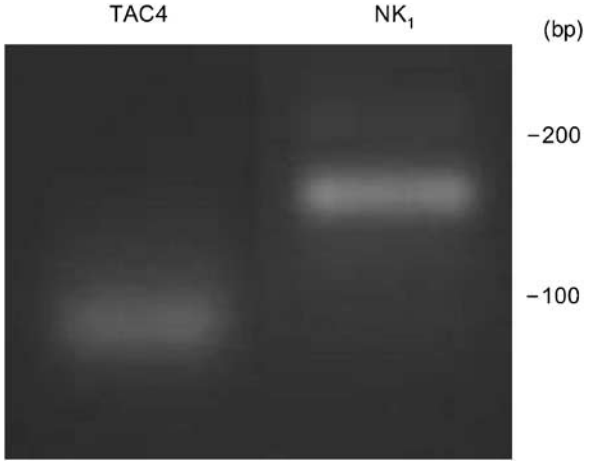

Figure 4 A representative gel of the PCR products for TAC4 and NK_1 mRNAs in microglial cells *in vitro*. The sizes of the PCR products for the TAC4 and NK_1 mRNAs are 62 and 148 bp, respectively.

expression levels on the ipsilateral side at day 3 (TAC1, 105.07 ± 18.96% expressed as a percentage of the value for the contralateral side, $P=0.81$; TAC4, 101.70 ± 6.81%, $P=0.82$; $n=4$).

TAC4 mRNA expression in microglial cells

Next, using a cultured microglial cell line, GMI-RI, we determined whether microglial cells express TAC4 mRNA. As shown in Figure 4, RT-PCR analysis revealed that TAC4 mRNA was expressed in this microglial cell line. In addition, mRNA expression of NK_1, a high affinity receptor for HK-1 and SP, was observed in the same cell line.

Effects of minocycline on the behaviour, microglial activation and spinal cord TAC4 mRNA expression of CCI rats

We further examined the effects of minocycline, an inhibitor of microglial activation, on rats treated with CCI. In the behavioural experiments, repeated administration of minocycline after the CCI operation inhibited the development of both mechanical allodynia and thermal hyperalgesia in CCI rats on day 3 ($n=5$; Figure 5a). Saline administration alone had no effect on neuropathic pain development ($n=5$; Figure 5a). Immunohistochemistry revealed that CCI increased the number and fluorescence intensity of CD11b-immunoreactive cells in the ipsilateral dorsal horn of the L4 spinal cord ($n=3$; Figure 5b) on day 3. These CD11b-positive cells also changed their morphology from ramified to amoeboid, indicating that microglial activation was induced by the CCI operation. Minocycline treatment blocked the activation of microglial cells in the ipsilateral dorsal horn of the L4 spinal cord on day 3 ($n=3$; Figure 5b). Intraperitoneal injection of saline or minocycline had no apparent effects on the microglial cells on the contralateral side of the dorsal horn, as evaluated by immunohistochemistry. In association with the suppression of both neuropathic pain development and microglial activation by minocycline, the above-described increase in TAC4 mRNA expression observed after the CCI operation was abolished in the ipsilateral L4 dorsal spinal cord of rats treated with CCI and minocycline on day 3 after CCI compared with that in rats treated with CCI and saline (CCI and saline-treated rats, 247.51 ± 31.91% expressed as a percentage of the value for the contralateral side; CCI and minocycline-treated rats, 172.0 ± 28.59%; $n=6$; $P<0.05$; Figure 5c). On the contralateral side of the dorsal spinal cord of CCI rats, minocycline treatment had no effect on the level of TAC4 mRNA expressed. In contrast to its effect on the expression of TAC4 mRNA in the spinal cord, minocycline did not suppress the increase in TAC1 mRNA expressed in the L4/L5 DRGs on day 3 after CCI compared with saline treatment (saline treatment, 114.66 ± 5.70% expressed as a percentage of the value for the contralateral side, $n=12$; minocycline treatment, 118.99 ± 6.34%, $n=12$; $P=0.62$; Figure 5c).

Discussion and conclusion

Differential expression of TAC1 and TAC4 mRNAs in the neuropathic pain state

In the present study, we have demonstrated differences in the mRNA expressions of TAC1 and TAC4 during the development of a neuropathic pain state. Specifically, TAC4 mRNA expression was increased in the dorsal spinal cord, but not the DRGs, on day 3 after CCI, whereas TAC1 mRNA expression was significantly increased in the DRGs, but not the dorsal spinal cord, on day 3 after CCI.

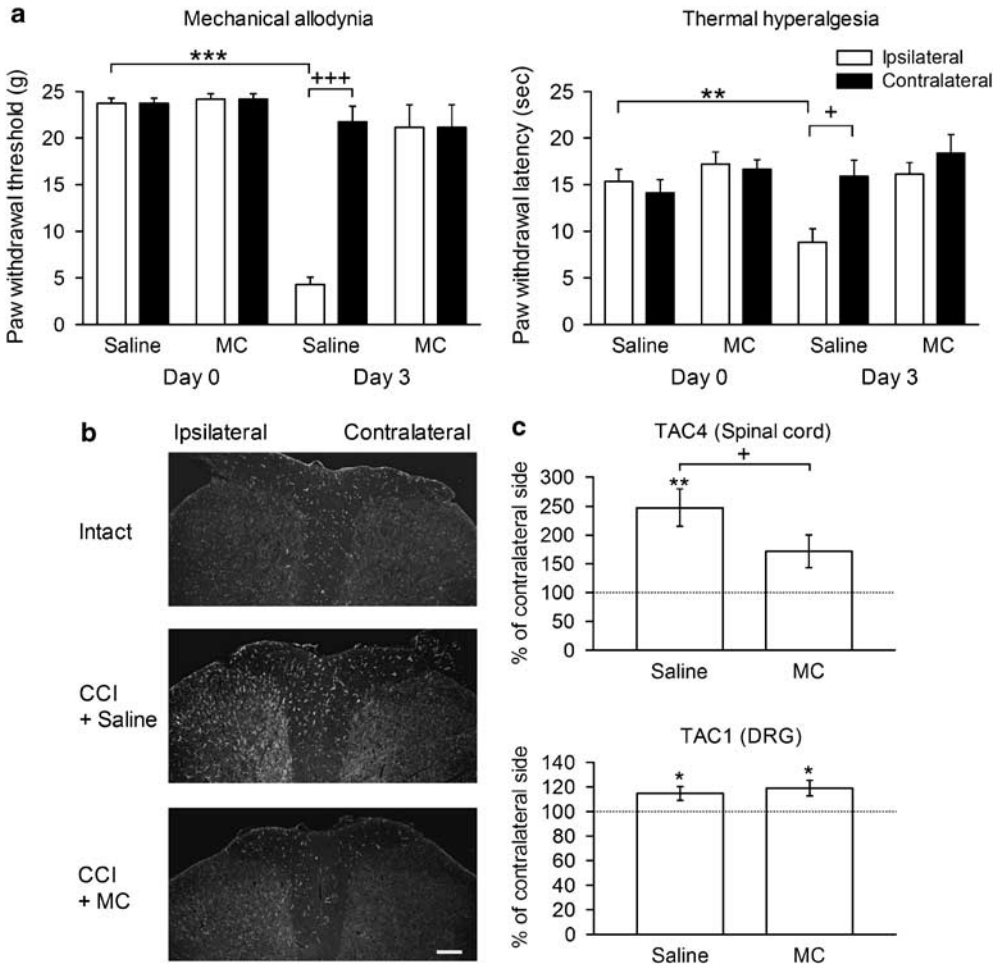

Figure 5 Effects of minocycline on rats at day 3 after the CCI operation. Minocycline hydrochloride, an inhibitor of microglial activation, was administered to CCI rats, i.p., every 12 h for 3 consecutive days. Saline alone was administered to CCI rats as a control. (**a**) The thresholds of paw withdrawal on the ipsilateral side and contralateral side in response to mechanical (left panel) or thermal (right panel) stimuli were measured in CCI rats treated with minocycline (MC) before (day 0) and 3 days after (day 3) the CCI operation. $^{**}P<0.01$ and $^{***}P<0.001$ vs the values on day 0 by one-way ANOVA, followed by Dunnett's *post hoc* test; $^{+}P<0.05$ and $^{+++}P<0.001$ vs the values on the contralateral side at the corresponding days by paired *t*-test; $n=5$. (**b**) Representative images of immunohistochemistry for activated microglial cells in the L4 spinal dorsal horn. L4 spinal cords were obtained from intact rats, as well as rats treated with CCI and saline (CCI + saline) and rats treated with CCI and minocycline (CCI + MC) at day 3 after CCI. The spinal cords were stained with an anti-CD11b antibody (OX-42), a microglial activation marker. Scale bar, 50 μm. The experiments for each treatment were repeated in three rats. (**c**) Real-time PCR analysis for TAC4 mRNA expression in the dorsal spinal cord (top) and TAC1 mRNA expression in the DRGs (bottom). Data are presented as percentages of the levels on the contralateral side. $^{*}P<0.05$ and $^{**}P<0.01$ vs the values of the contralateral side by the paired *t*-test; $n=6$ (spinal cord) and $n=12$ (DRG). $^{+}P<0.05$ vs the values in the saline-treated rats.

Consistent with our present observations, increased expression levels of SP peptide and its mRNA (TAC1) in the DRGs have been observed in the early phase after nerve injury (Marchand *et al*., 1994). SP released from central axon terminals of DRG neurons in the spinal cord binds to NK_1 receptors expressed in nociceptive projection neurons, and sensitizes these neurons to cause hyperalgesia (Basbaum, 1999; Nichols *et al*., 1999; Snijdelaar *et al*., 2000). HK-1 can also bind to NK_1 receptors with a similar potency to SP (Kurtz *et al*., 2002). Therefore, in addition to SP, HK-1 produced in the spinal cord may contribute to such sensitization of second-order nociceptive neurons. In fact, Endo *et al*. (2006) demonstrated that intrathecal administration of HK-1 to naive rats induced a pain-related behaviour (scratching), which was similar to the effect of SP administration. On the other hand, they also showed that intrathecal SP administration evoked another pain-related behaviour (thermal hyperalgesia), whereas HK-1 did not (Endo *et al*., 2006). Although the mechanisms underlying this discrepancy in the effects of SP and HK-1 on nociception are still unclear, the existence of innate receptors for HK-1 other than NK_1 receptors has been suggested as a possible explanation (Zhang and Paige, 2003; Naono *et al*., 2007). Collectively, these two tachykinin family members, HK-1 and SP, may provide different contributions to neuropathic pain. This possibility may be worthy of further investigation from the point of view of developing anti-nociceptive drugs, as NK_1 antagonists have so far failed to produce analgesia in clinical trials (Rupniak and Kramer, 1999; Hill, 2000).

In the present study, we were unable to confirm which cells expressed TAC4 mRNA in the spinal cord, as the level of TAC4 mRNA expression was lower than the detection limit

of the non-radioisotopic *in situ* hybridization method adopted in our preliminary experiments. It is also unclear whether the translated HK-1 peptide is indeed present and increased in the spinal cord in the neuropathic pain state, as no specific antibodies against HK-1 are available. Therefore, further studies are needed to confirm the role of HK-1 in neuropathic pain.

TAC4 mRNA expression in microglial cells

Hemokinin-1 was originally identified as a regulator of B lymphopoiesis and is thought to act in an autocrine and paracrine manner (Zhang *et al*., 2000). SP expression is almost completely restricted to the nervous systems, whereas HK-1 is expressed not only in the nervous systems but also in many peripheral organs and immunocompetent cells, such as microglial cells, macrophages and dendritic cells (Kurtz *et al*., 2002; Duffy *et al*., 2003; Nelson *et al*., 2004). In the present study, we demonstrated that microglial cells expressed TAC4 and NK_1 mRNAs *in vitro*. Consistent with these findings, the increase in TAC4 mRNA expression in the spinal cord after CCI was effectively blocked by i.p. administration of minocycline, which inhibits microglial activation both *in vivo* and *in vitro* (Tikka and Koistinaho, 2001; Tikka *et al*., 2001; Wang *et al*., 2005). This blocking effect of minocycline seemed to be specific for TAC4 mRNA expression in the spinal cord, as the increase in TAC1 mRNA expression in the L4/L5 DRGs on day 3 after CCI was not affected by the minocycline treatment. Taken together, these results suggest that the increased expression of TAC4 mRNA in neuropathic pain occurs at least partly in activated microglial cells. Recently, the dynamic and active roles of microglial cells have been studied (Hanisch and Kettenmann, 2007). Upon peripheral nerve injury, microglial cells in the spinal cord change from their resting to their reactive phenotype (Tsuda *et al*., 2005) and secrete a variety of molecules, thereby contributing to neuropathic pain (Tsuda *et al*., 2005; Hanisch and Kettenmann, 2007). Activation of spinal microglial cells has been shown to peak at around day 3 after peripheral nerve injury (Gehrmann and Banati, 1995) and to be crucial for the development of neuropathic pain (Jin *et al*., 2003; Milligan *et al*., 2003; Zhuang *et al*., 2007). It is noteworthy that, in the present study, the increase in TAC4 mRNA expression in the dorsal spinal cord was observed on day 3 in association with the development of pain, and then declined by day 7 after nerve injury. Therefore, HK-1 may be secreted by activated microglial cells and play a role in the early phase of a neuropathic pain state.

Given that HK-1 is secreted by activated microglial cells, it could act on NK_1 receptor-bearing cells. As discussed above, NK_1 receptors are expressed on nociceptive spinothalamic neurons in spinal cord laminae I and V (Nakaya *et al*., 1994), and the increased HK-1 may, therefore, sensitize these projection neurons in concert with SP, resulting in a modification of pain perception. Alternatively, microglial cells may be another target of HK-1. Microglial cells themselves express NK_1 receptors as shown in the present and a previous study (Pocock and Kettenmann, 2007). SP has been proposed to activate the transcriptional factor NF-κB (Rasley *et al*., 2002) and NADPH oxidase (Block *et al*., 2006) in microglial cells through the NK_1-mediated pathway. Therefore, HK-1 released from microglial cells appears to act in an autocrine and/or paracrine manner, as observed in B lymphopoiesis (Zhang *et al*., 2000). Additional studies are required to investigate these possibilities further.

To conclude, our results suggest that HK-1 plays a role in neuropathic pain, which is different from that of the conventional tachykinin SP and occurs in a different place. Therefore, a comprehensive assessment of the contributions of the various tachykinins to neuropathic pain would be useful for the development of more effective tachykinin-related analgesics.

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Conflict of interest

The authors state no conflict of interest.

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