What's already known about this topic?
- Previous studies reported that platelet-activating factor receptor (PAF-R) antagonists have varied anti-allodynia effects in rat nerve injury.

What does this study add?
- Profound anti-allodynia effects by systemic injection of PAF-R antagonists were found or by intrathecal injection of siRNA of PAF-R in four different nerve injury models in mice.
- The results included long-lasting effects with initially competitive and the following non-competitive manner of inhibition.

1. Introduction

Several lines of evidence support the role of platelet-activating factor (PAF) as an important mediator of inflammatory responses (Ishii and Shimizu, 2000; Doi et al., 2006). However, the relevance of PAF in the pathology of pain is less clear, although some studies showed that PAF injected into the rat hind paw caused hypersensitivity to noxious stimulation (Bonnet et al., 1981; Vargaftig and Ferreira, 1981; Dallob et al., 1987; Marotta et al., 2009), persistent pain behaviours resulting from tissue injury by locally injected formalin or capsaicin were reduced in mice lacking the PAF receptor (Tsuda et al., 2007).

We have previously suggested that PAF may be a mediator of neuropathic pain. PAF injection into the mice spinal cord caused thermal hyperalgesia and tactile allodynia; key symptoms of neuropathic pain arise from innocuous stimuli, which were blocked by PAF receptor antagonists (Morita et al., 2004, 2008a). Subsequent studies showed PAF receptor blockade reduced pain behaviours elicited in nerve injury models. A PAF receptor antagonist CV-3988 by injecting near the dorsal root ganglion (DRG) in rats or mice lacking PAF receptors showed a reduction in tactile allodynia following spinal nerve injury accompanied by suppression of up-regulation of tumour necrosis factor and interleukin-1β expression in the injured DRG (Hasegawa et al., 2010). DRG contains lyso-PAF-acetyltransferase/lysophosphatidylcholine acyltransferase 2 (LPCAT2) and PAF receptor mRNA was increased in the ipsilateral DRG after nerve injury (Hasegawa et al., 2010). LPCAT2 mRNA and PAF receptor mRNA were increased in spinal microglia after nerve injury in rat spared nerve injury (SNI) model and i.t. injection of PAF antagonist WEB 2086 until 9 days post surgery suppressed the development of mechanical allodynia in the model (Okubo et al., 2012). PAF has been implicated in the pathophysiology of delayed tissue damage after various forms of brain injury including ischaemia, hypoxia and trauma (Lindsberg et al., 1990; Faden and Halt, 1992; Hostettler and Carlson, 2002). The evidence suggests that PAF contributes to neural tissue damage and pain behaviour after nerve injury.

However, neuropathic pain develops diverse pathological profiles depending on the disease, and thus the effectiveness of treatment with drugs is not uniform among them. Delayed administration of WEB 2086 did not reverse the allodynia in rat SNI model (Okubo et al., 2012).

CV-3988 did not suppress allodynia by injecting it into the lumbar enlargement of the spinal cord, while it was suppressed by injection near L5 DRG in spinal nerve-injured rats (Hasegawa et al., 2010). Therefore, we examined the effectiveness of systemic administration of several PAF antagonists against neuropathic pain in several different models in mice including a partial sciatic nerve ligation injury model, a partial infraorbital nerve ligation model, a chronic constriction of the infraorbital nerve injury (CCI model) and streptozotocin (STZ)-induced diabetes model, and found that PAF antagonists, TCV-309, BN 50739 and WEB 2086 produced profound and long-lasting anti-allodynia effects in these models. We further investigated the mode of action of PAF antagonist-induced pain relief.

2. Materials and methods

2.1 Animals

Experiments were performed on adult male ddY mice (Kyudo, Kumamoto, Japan) that were 5 weeks old and weighed 25–30 g at the beginning of the study. Mice were housed at 22 ± 2 °C with free access to commercial food and tap water. All experimental procedures and animal handling were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the guidelines of Hiroshima University, Hiroshima, Japan. The animals were used for only one measurement in each experiment.

2.2 Animal models

2.2.1 Preparation of partial sciatic nerve injury mice and painful diabetic neuropathy mice

The experiments were conducted using a previously reported methods (Morita et al., 2008b).

2.2.2 Infraorbital nerve injury mice

Mice were anaesthetized with pentobarbital (60 mg/kg, i.p.). The head of the mouse was fixed in a Narishige head holder and mounted in a Kopf stereotaxic frame. A midline scalp incision was made, exposing the skull and nasal bone. The edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free. To give access to the infraorbital nerve, orbital contents were gently deflected with a cotton-tripped wooden rod. The infraorbital nerve was dissected free at its most rostral extent in the orbital cavity, just caudal to the infraorbital foramen. Partial ligation of the infraorbital nerve was performed by tying the distal one-third to one-half of the infraorbital nerve (Partial ligation model). Chronic constriction of the infraorbital nerve was performed by loosely tying around the infraorbital nerve (2 mm apart) using two chromic gut (6-0) ligatures. To obtain the desired degree of constriction, a criterion formulated by Bennett and Xie (1988) was applied: the ligations reduced the diameter of the nerve by a just noticeable amount and retarded it, but did not occlude circulation through the superficial vasculature (CCI model). The scalp incision was closed in layers using nylon sutures. In mice in the control group, the infraorbital nerve was exposed on one side using the same procedure. The exposed infraorbital nerve was not ligated. Tactile allodynia was evaluated by measuring the face withdrawal threshold in response to probing with a series of calibrated fine filaments. Two weeks following nerve ligation, von Frey withdrawal thresholds were measured and the mice received a single dose a PAF antagonist or a vehicle.

2.3 Measurement of touch-evoked tactile allodynia

Tactile allodynia was assessed by lightly stroking the injured leg with a paintbrush or evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments as reported previously (Morita et al., 2008a,b).

2.4 Knockdown of PAF receptor and GlyRα3 in the spinal cord

Knockdown of PAF receptor and GlyRα3 were performed according to previous report (Morita et al., 2008a). The hemagglutinating virus of the Japan (HVJ) envelope vector system (HVJ Envelope Vector Kit GenomONE; Ishihara Sangyo Kaisha, Ltd, Osaka, Japan) was used for *in vivo* siRNA transfer. This HVJ-Envelope Vector has been proven to be an effective oligodeoxynucleotide delivery system both *in vitro* and *in vivo* (Kaneda et al., 2002). siRNAs were incorporated into the HVJ-Envelope Vector according to the manufacturer's instructions. Briefly, after mixing 40 μl (1 assay unit, AU) of HVJ-Envelope Vector with 4 μl of the enclosing factor, the mixture was centrifuged (10,000 × *g*, 10 min, 4 °C), and the pellet suspended in 10 μl of buffer solution. Then, 10 μl of a mixture of three siRNAs solution (#1, #2 and #3, 1 μg/μl each) was added, and the mixture was kept on ice for 5 min. Sterile artificial cerebrospinal fluid (ACSF, 10 μl) containing synthetic siRNA duplexes (0.45 pmole/animal) was injected into the subarachnoid space between the L5 and L6 vertebrate of conscious mice. The sequences of the siRNA oligonucleotide (sense) were as follows: PAF receptor (#1, 5′-CACCUCAGUGAGAAGUUUUACAGCA-AG-3′; #2, 5′-ACCCUUCCAAGAAACUAAAUGAGAU-AG-3′; #3, 5′-CAACUUCCAUCAGGCUAUUAAUGAU-AG-3′; targeting sequences around position 1005 to 1029, 232 to 256, 893 to 917, respectively, in *pafr*, GenBank accession no. D5087); GlyRα3 (#1, 5′-AGGUUUCGGCGAAAGAGAAAGAAUA-AG-3′; #2, 5′-GGUACUGCACUAAACACUACAAUAC-AG-3′; #3, 5′-CCUUAGGCAUGAAGACAUUCAUCAU-AG-3′; targeting sequences around position 1124 to 1148, 765 to 789, 1438 to 1462, respectively, in *glyra3*, GenBank accession no. NM_080438). Moreover, mismatched siRNA with three or four nucleotide mismatches was prepared to examine nonspecific effects of siRNA duplexes (PAF receptor siRNA#4, 5′-ACUCUGCCAAGAGACUACAUGAGAU-AG-3′; GlyRα3 siRNA#4, 5′-GAUACUGCACUACACACUACGAUAC-AG-3′). These selected sequences were also submitted to a BLAST search (Bioinformatics Center Institute for Chemical Research, Kyoto University, Japan) against the mouse genome sequence to ensure that only one gene in the mouse genome was targeted. siRNAs were purchased from iGENE Therapeutics Inc. (Tsukuba, Japan).

2.5 Experimental procedures

The first experiment examined whether various PAF antagonist had a profound anti-allodynia action in the present experimental neuropathic pain model. Following habituation and baseline testing, all mice received nerve injury as described above and the tactile allodynia was evaluated using paintbrush and von Frey tests. On a given test day, tactile allodynia was performed in all mice, and those displaying similar levels of tactile allodynia were randomized then divided into several groups. Mice then received intravenous (i.v.), i.t. or oral (per os, p.o.) injection of TCV-309, BN 50739, WEB 2086 or an equivalent volume of vehicle for drugs. Tactile allodynia was assessed before and after the i.t., i.v., p.o, injection.

A separate group of mice was used in the second experiment to examine the mode of anti-allodynia action of TCV-309 *in vivo*. Following habituation and four baseline measurements, mice were divided into several groups based on their responses to paintbrush and von Frey stimulation, providing those groups with similar average baseline mechanical sensitivity. On a given test day, TCV-309 (0.1 mg/kg) was intravenously injected 30 min after i.t. injection of 10 pg, 0.1 ng or 1 ng/mouse of PAF. The effect of TCV-309 pretreatment on allodynia induced by PAF was also examined. TCV-309 (0.1 mg/kg) was intravenously injected 30 min or 3 days before i.t. injection of various doses (0.001–

1000 pg/mouse) of PAF. Tactile allodynia was assessed before and after the i.t., i.v. injection.

Another group of mice received spinal transfer of siRNA against the PAF receptor or GlyRα3 to examine the involvement of the spinal PAF receptor and GlyRα3 in the PAF antagonist-induced anti-allodynia action in neuropathic pain models. Following habituation and baseline testing, all mice received nerve ligation as described above and the emergence of mechanical hypersensitivity was measured. On day 12 post surgery, tactile allodynia was performed in all mice, and those displaying similar levels of tactile allodynia were randomized then divided into several groups. Mice then received i.t. injection of either siRNAs, mutant siRNA, or HVJ-E vector alone. The effect of knockdown of the spinal PAF receptor on established nerve injury assessed tactile allodynia was examined. At 3 days after the knockdown of GlyRα3 by siRNA, the anti-allodynia response to TCV-309 was examined. The effect of a prophylactic PAF antagonist and knockdown of the spinal PAF receptor on the development of nerve injury tactile allodynia was also examined. In the prophylactic paradigm, following habituation and baseline testing, mice were divided into several groups based on their responses to both paintbrush and von Frey stimulation, providing those groups with similar baseline mechanical sensitivity. Mice then received i.v. injection of TCV-309 (100 μg/kg), BN 50739 (100 μg/kg), WEB 2086 (100 μg/kg) or a vehicle, or i.t. injection of siRNA or a mutant siRNA of PAF receptor mRNA or an HVJ-E vector alone 30 min or 3 days before surgery (at time 0). Tactile allodynia was assessed before and after the nerve ligation. Knockdown of the PAF receptor or GlyRα3 protein after spinal transfection of the PAF receptor or GlyRα3 siRNA were confirmed by immunoblotting analysis.

Throughout the experiment, behavioral testing was performed under blind conditions by a single experimenter. Injections were performed blind by another person.

2.6 Materials

PAF (1-*O*-hexadecyl-2-acetl-*sn*-glycero-3-phosphocholne), gabapentin (1-(aminomethyl)-cyclohexaneacetic acid) and STZ were obtained from Sigma-Aldrich (St. Louis, MO, USA). WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo- [4,3-*a*][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone) was obtained from Tocris Bioscience (Ellisville, MO, USA). TCV-309 (3-bromo-5-[*N*-phenyl-*N*-[2-[[2-(1,2,3,4-tetrahydro-2-isoquinolyl- carbonyloxy)ethyl]carbamoyl]ethyl]carbamoyl]-1-propylpyridinium nitrate), and BN 50739 (tetrahydro-4,7,8,10methyl-(chloro-2phenyl)6[dimethoxy-3,4-phenylthio]methylthiocarbonyl-9 pyrido[4′,3′-4,5]thieno[3,2-*f*]triazolo-1,2,4[4,3-*a*]diazepine-1,4) were donated from Takeda Pharmaceutical Co., and Institute Henri Beaufour, respectively.

BN 50739 was dissolved in a solvent containing 25% 2-hydroxypropyl-β-cyclodextrin (Sigma/RBI, Natick, MA, USA) and distilled water, pH adjusted to ~6 using 1 N NaOH, and diluted appropriately with ACSF or saline. PAF was dissolved in ethanol, which was then removed from an aliquot of this solution in a siliconized tube by introducing nitrogen gas into the tube. The PAF was then dissolved in 0.05% fatty acid-free bovine serum albumin containing ACSF. Other reagents were dissolved in ACSF or saline. ACSF composition (in mM) was NaCl 142, KCl 5, $CaCl_2 \cdot 2H_2O$ 2, $MgCl_2 \cdot 6H_2O$ 2, NaH_2PO_4 1.25, D-glucose 10, HEPES 10, pH 7.4.

2.7 Data analysis

Data are expressed as the mean ± standard error of the mean (SEM) for each treatment group. Values are presented as an average allodynia score or withdrawal threshold at each time point during the time-course study. In other studies, allodynia was assessed every 5 min over a 60-min period (12 trials) and the values were expressed as the average % maximum possible cumulative score (possible cumulative maximum score: 2/mouse × 12 trials = 24), as described previously (Morita et al., 2004).

Regarding the statistical analyses of tactile allodynia, comparisons of allodynia scores and the withdrawal threshold of the differences between the drug-treated groups and the vehicle-treated group were evaluated using Dunnett's Multiple Comparison Test or unpaired *t*-test using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Effects of systemic administration of TCV-309, BN 50739 and WEB 2086, specific PAF receptor antagonists, on tactile allodynia in various neuropathic pain models of mice

Effects of PAF antagonists on allodynia induced by partial sciatic nerve ligation injury, partial infraorbital nerve ligation, CCI model and STZ-induced diabetes in mice were examined. TCV-309 with a range of 10~100 μg/kg by i.v and 30 μg~1 mg/kg by p.o. dose-dependently increased the withdrawal threshold at 10 to 15 days after partial ligation of the sciatic nerve in mice (Fig. 1A and B). The effects of TCV-309 were quite long lasting, for instance, TCV-309 ameliorated mechanical allodynia by a single injection of 10, 30 or 100 μg/kg, i.v. with a peak effect at 1 to 2 days after the injection until 3, 5 and 7 days, respectively. TCV-309 up to 1 mg/kg did not affect general behaviour or motor function estimated by RotaRod test (data not shown). BN 50739 and WEB 2086 also produced a long-lasting anti-allodynia effect with a similar range of doses (Table 1). The effect of these antagonists were much more potent and long lasting in comparison with the anti-allodynia effect of gabapentin 75 mg/kg, i.v. for which the anti-allodynia effect disappeared

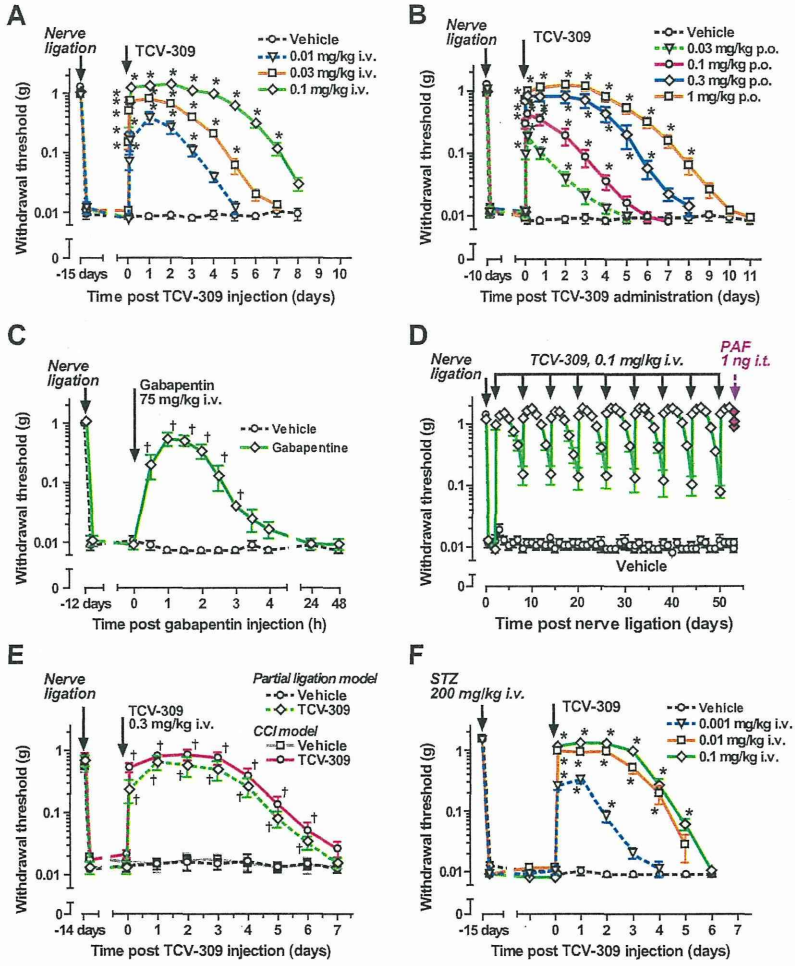

Figure 1 (A, B) Systemic administration of TCV-309 ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 was administered intravenously (A) or orally (p.o., B) at time 0. Experiments were performed at 10–15 days after the nerve ligation. Data are expressed as the mean ± standard error of the mean (SEM) $n = 7$–15 mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatments. $^*p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test. (C) Intravenous injection of gabapentin ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Gabapentin (75 mg/kg) was administered intravenously at time 0. Experiments were performed at 12 days after the nerve ligation. Data are expressed as the mean ± SEM $n = 7$ mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatments. $\dagger p < 0.01$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (D) Reproduction of the anti-allodynia effect of TCV-309 by repeated injection of TCV-309 in partial sciatic nerve ligation injury mice. TCV-309 was injected intravenously at 2, 8, 14, 20, 26, 32, 38, 44, 50 days after surgery. Tactile allodynia was assessed in mice using von Frey hairs (◊) on the ipsilateral paws 3 h and 1, 2 and 3 days after TCV-309 injection. Platelet-activating factor was administered intrathecally 3 days after the last injection of TCV-309. Preoperative basal values were obtained on day 0. Data are expressed as the mean ± SEM $n = 10$ mice per group. Control mice received injections with a vehicle: saline. (E) Intravenous injection of TCV-309 ameliorated tactile allodynia that developed in partial infraorbital nerve ligation model and chronic constriction of the infraorbital nerve injury (CCI) model mice. The experiments were carried out 14–20 days after the infraorbital nerve injury. Tactile allodynia was evaluated by measuring the face withdrawal threshold in response to probing with a series of calibrated fine filaments. TCV-309 (0.3 mg/kg) was administered intravenously at time 0. Data are expressed as the mean ± SEM $n = 6$–10 mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after infraorbital nerve ligation injury in mice was not affected by vehicle treatment. $\dagger p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (F) Intravenous injection of TCV-309 ameliorated tactile allodynia developed in streptozotocin (STZ)-induced painful diabetic neuropathy mice. The experiments were carried out 10–40 days after the injection of STZ or a vehicle. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 was administered intravenously at time 0. Data are expressed as the mean ± SEM $n = 13$ mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after STZ injection in mice was not affected by vehicle treatment. $^*p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test.

Table 1 Effects of platelet-activating factor antagonists on tactile allodynia in various neuropathic pain models in mice.

	Paintbrush test				Von Frey test			
	ED_{50} (μg/kg)		Duration (days)		ED_{50} (μg/kg)		Duration (days)	
			0.1 μg/kg	0.3 μg/kg			0.1 μg/kg	0.3 μg/kg
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Nerve ligation model								
TCV-309	7.2	50.0	7	7	10.8	110.0	8	7
BN 50739	13.0	29.6	7	7	14.5	33.4	8	7
WEB 2086	4.5	30.6	6	8	8.4	67.1	6	8
STZ diabetic model								
TCV-309	1.5	16.0	5	7	5.8	38.0	5	7
BN 50739	30.0	77.0	5	6	110.0	125.0	6	6
WEB 2086	17.0	100.0	5	5	67.0	200.0	6	5

Nerve ligation model: partial sciatic nerve ligation injury model. STZ diabetic model: streptozotocin (STZ, 200 mg/kg i.v.)-induced painful diabetic neuropathy model. ED_{50} values were estimated from the peak response by using least-squares linear regression. Duration: values are time during significant reduction of allodynia score and rise of withdrawal threshold. Data are expressed as the mean (n = 7–15 mice per group).

within 4 h after i.v. injection (Fig. 1C). As the anti-allodynia effect of TCV-309 0.1 mg/kg, i.v. lasted for 7 days, the effect of the repeated administration every 6 days was examined. The potency of TCV-309 was similar in nine trials over 50 days without any refractory effect (Fig. 1D). I.t. injection of PAF at 1 ng produced a marked allodynia response (data not shown). When PAF was injected 3 days after the injection of the last TCV-309, it failed to produce allodynia, suggesting the effectiveness of TCV-309 as a PAF antagonist. The results show the PAF antagonists have potent anti-allodynia effects at very low doses and can avoid repeat treatment. However, one difficulty of clinically treating neuropathic pain with drugs is the difference in the effectiveness of drugs on different causes of pain. Therefore, the effectiveness of PAF antagonists on mechanical allodynia in other models such as a partial infraorbital nerve ligation model, a CCI model and a STZ-induced diabetes model were examined. The withdrawal threshold constantly decreased 10 days after the operation in both the partial infraorbital nerve ligation model and CCI model. Fourteen days after the operation, TCV-309 at 0.1 mg/kg, i.v. effectively increased the withdrawal threshold in both partial infraorbital nerve ligation model and the CCI model shortly after the injection and the effect lasted over 5 days (Fig. 1E). The anti-allodynia action of TCV-309, BN 50739 and WEB 2086 by i.v. injection in the STZ-induced diabetic model is shown in Fig. 1F. Anti-allodynia effects were obtained with a similar order of drugs in the nerve ligation models. The ED_{50} values of TCV-309, BN 50739 and WEB 2086 administered by the i.v. and p.o. routes are summarized in Table 1. In the diabetic neuropathic model, TCV-309 was the most effective of the three drugs as shown by the low ED_{50} value assessed by either the allodynia score or withdrawal threshold. However, the rank order of these drugs varied with the model and route of administration.

3.2 Effects of blockade of spinal PAF receptors on allodynia induced by partial ligation of the sciatic nerve and STZ-induced diabetes in mice

To elucidate the site of anti-allodynia action of PAF receptor antagonists, effects of i.t. injection of TCV-309, BN 50739 and WEB 2086, and also interference in the expression of spinal PAF receptors using siRNA of PAF receptor mRNA were examined. Spinal administration of 10 pg of these drugs immediately ameliorated allodynia (Fig. 2A). The evidence suggests that the site of action of PAF antagonists to produce an anti-allodynia effect involves at least the level of spinal cord. Knockdown of the expression of spinal PAF receptor protein was achieved by intrathecal transfer of PAF receptor siRNA in mice. As previously reported, a significant reduction in PAF receptor expression in normal mice and mice 15 days after surgery on the sciatic nerve achieved 37.5 ± 5.4% and 29.9 ± 4.9% of the control at 3 days after siRNA transfection, respectively, while the expression was not altered by the HVJ-E vector alone or mutant siRNA (Morita et al., 2008a). I.t. injection of PAF (0.1 pg) failed to evoke allodynia in siRNA-treated mice, while it induced allodynia in mice treated with mutant siRNA and the HVJ-E vector, as well as in vehicle-treated mice (these data are not shown). Thus, the knockdown of PAF receptor function by siRNA was established. The withdrawal threshold increased with the peak effect at 2 to 3 days after siRNA transfection,

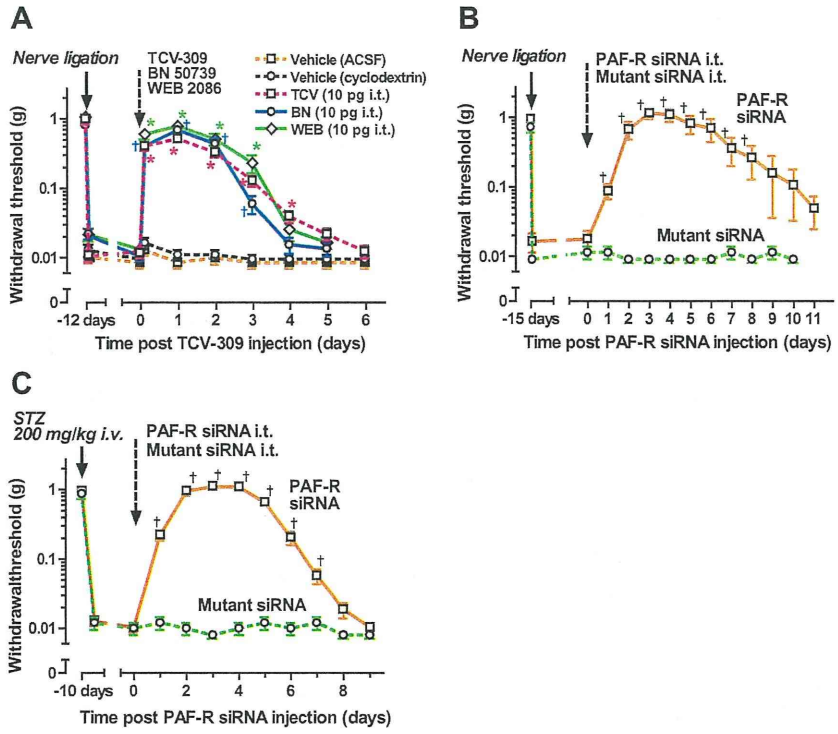

Figure 2 (A) Intrathecal administered of platelet-activating factor (PAF) antagonists ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 (10 pg/mouse), BN 50739 (10 pg/mouse), WEB 2086 (10 pg/mouse) or their vehicle were injected intrathecally at time 0. Experiments were performed at 12 days after the nerve ligation. Data are expressed as the mean ± standard error of the mean (SEM) $n = 6$–12 mice per group. Control mice received injections with a vehicle: ACSF or 25% 2-hydroxypropyl-β-cyclodextrin (cyclodextrin). Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatment (not shown). *$p < 0.05$ compared with the corresponding control [vehicle (ACSF) injection] values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test. †$p < 0.01$ compared with the corresponding control (vehicle (cyclodextrin) injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (B) Knockdown of spinal PAF receptor by siRNA ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed after partial sciatic nerve ligation injury in mice using von Frey hairs on the ipsilateral paws. siRNA or mutant siRNA of PAF receptor (PAF-R) mRNA were transfected into the spinal cord 15 days after nerve ligation. Data are expressed as the mean ± SEM ($n = 6$–8 mice per group). †$p < 0.05$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (C) Knockdown of spinal PAF receptor by siRNA ameliorated tactile allodynia that developed in streptozotocin (STZ)-induced diabetic mice. Tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with von Frey hairs. siRNA of PAF receptor (PAF-R) mRNA were transfected into the spinal cord 10 days after the treatment with STZ. Data are expressed as the mean ± SEM $n = 10$ mice per group. †$p < 0.01$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test.

while the anti-allodynia action gradually disappeared over 9 days in sciatic nerve-injured mice (Fig. 2B). Injection of mutant siRNA had no effect on the development of allodynia (Fig. 2B). Knockdown of the PAF receptor also reduced the allodynia response in STZ-induced neuropathic mice (Fig. 2C). The results further support the spinal site of anti-allodynia action of the PAF receptor blockade.

3.3 Effects of spinal GlyRα3 knockdown

We previously reported that intrathecally injected PAF-induced allodynia was mediated through glutamate-NO-cGMP and that this effect was blocked by knockdown of spinal GlyRα3. Therefore, the effects of knockdown of spinal GlyRα3 on the expression of tactile allodynia and PAF antagonist-induced anti-allodynia action were examined (Fig. 3). Fourteen days after sciatic nerve ligation, the allodynia score was maximally developed (maximal score is 2) and thus further increases in the score were not possible by transfection of GlyRα3 siRNA (Fig. 3A). TCV-309 at 100 μg/kg, i.v., more than the largest dose to produce an anti-allodynia effect in naïve animals (Fig. 1A) failed to ameliorate the allodynia in mice transfected with GlyRα3 siRNA, while it produced a profound

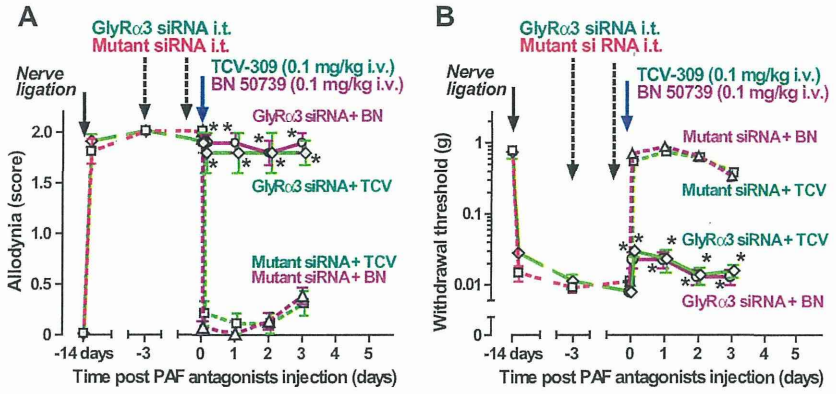

Figure 3 Effect of knockdown of spinal GlyRα3 by siRNA on the anti-allodynia effect of PAF antagonists. Tactile allodynia was assessed after partial sciatic nerve ligation injury in mice using a paintbrush (A) or von Frey hairs (B) on the ipsilateral paws. siRNA (solid line) or mutant siRNA (dotted line) of GlyRα3 mRNA were transfected into the spinal cord 3 days and 3 h before the intravenous injection of TCV-309 (0.1 mg/kg) or BN 50739 (0.1 mg/kg). The experiments were carried out 11 days after nerve ligation. Data are expressed as the mean ± standard error of the mean $n = 10$ mice per group. $^*p < 0.01$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test.

anti-allodynia effect in mutant siRNA-treated mice (Fig. 3A). Transfection with GlyRα3 siRNA, but not mutant siRNA, slightly further reduced the withdrawal threshold and markedly reduced the anti-allodynia effect of TCV-309 (Fig. 3B). The anti-allodynia effect of BN 50739 was also reduced in GlyRα3 siRNA-transfected mice (Fig. 3B). Knockdown of GlyRα1 did not affect the effect of TCV-309 (data not shown). The evidence suggests that TCV-309 may exert its anti-allodynia action by antagonizing the reduction of GlyRα3-mediated inhibitory control of pain signal transduction by PAF in the spinal cord.

Glycine transporter (GlyT) inhibitors have a potent anti-allodynia effect in neuropathic pain models and spinal GlyRα3 has been implicated in the anti-allodynia action (Morita et al., 2008b; Dohi et al., 2009). However, the anti-allodynia action of GlyT inhibitors was stage-dependent on development of neuropathy, for instance, they did not block the developing allodynia response over 3 to 4 days after nerve injury and then produced a marked anti-alllodynia effect in established neuropathy in a partial sciatic nerve ligation model (Morita et al., 2008b). Although the present study showed the anti-allodynia effects by blocking PAF receptors in the established stage of neuropathy, whether or not blocking PAF receptors prior to surgery is effective was also examined. In control mice, almost a maximum allodynia score and a profound decrease in withdrawal threshold were observed shortly after nerve ligation. When PAF antagonists were injected intravenously 30 min before surgery, allodynia responses were not initiated until 4 days post surgery and allodynia gradually appeared and reached a stable state 8 days post surgery (Fig. 4A). The periods with suppressed appearance of allodynia by PAF antagonists post surgery almost corresponded with the effective periods of these antagonists. When treatment with PAF receptor siRNA was given 3 days before surgery, the initiation of allodynia started from 5 days post surgery, while it started shortly after surgery in mutant siRNA-treated mice (Fig. 4B). These results showed that PAF antagonists exert an anti-allodynia action against initiation of allodynia, as well as in an established state of allodynia.

3.4 Mode of anti-allodynia action of PAF antagonists

There is a striking difference in the duration of anti-allodynia action between PAF antagonists and gabapentin. To explore the long-acting effect of PAF antagonists, the dose-dependent mode of action of PAF antagonists against PAF-induced allodynia *in vivo* was analysed using TCV-309.

When TCV-309 (100 μg/kg, i.v.) was given at 30 min after 10 pg of PAF, i.t., the allodynia score decreased for 60 min after TCV-309 injection. The anti-allodynia potency of TCV-309 decreased by increasing concentration of PAF 10 pg to 1 ng of PAF (Fig. 5A). However, the anti-allodynia potency of TCV-309 gradually intensified as a function of time after TCV-309 treatment, for instance, the anti-allodynia potency of TCV-309 against PAF 1 ng was slight for 60 min, but gradually intensified and reached the maximum at 12 h after TCV-309 injection (Fig. 5A). These results suggest that TCV-309 has a different mechanism of action depending on the time after the injection. This concept is further supported

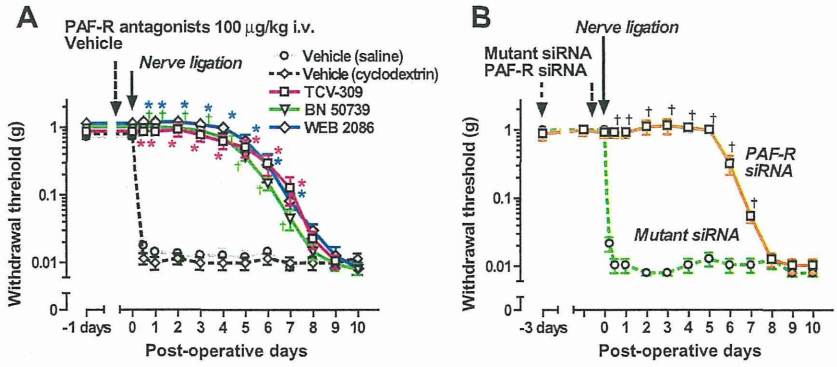

Figure 4 Effect of prophylactic platelet-activating factor (PAF) antagonists or spinal PAF receptor knockdown on the development of partial sciatic nerve ligation induced tactile allodynia in mice. Tactile allodynia was assessed in mice using von Frey hairs on the ipsilateral paws. PAF antagonists were administered i.v. 30 min before nerve ligation. Control mice received injections with a vehicle: saline or 25% 2-hydroxypropyl-β-cyclodextrin (cyclodextrin) (A). siRNA or mutant siRNA of PAF receptor (PAF-R) mRNA were transfected into the spinal cord 3 days and 1 h before surgery (B). Data are expressed as the mean ± standard error of the mean (n = 6–9 mice per group). *$p < 0.05$ compared with the corresponding control (vehicle (saline) injection) values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test. †$p < 0.01$ compared with the corresponding control (vehicle (cyclodextrin) or mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired t-test.

by the following analysis. Intrathecal PAF caused allodynia dose-dependently from 0.001 pg and the response reached a maximum at 0.03 pg (Fig. 5B). When TCV-809 (0.1 mg/kg, i.v.) was pretreated 20 min before PAF injection, the dose-response curve of PAF parallel shifted to the right and the maximum response was obtained at 1000 pg (Fig. 5B). However, when TCV-309 was pretreated at 3 days before the injection of PAF, increasing the dose of PAF up to 1000 pg did not overcome the decreased response. These results suggest that TCV-309 inhibits PAF-induced allodynia in a competitive manner at shortly after the injection of TCV-309 and in a non-competitive manner later.

4. Discussion

The present study showed that PAF antagonists, TCV-309, BN 50739 and WEB 2086 by i.v. or p.o. all have a potent anti-allodynia action in partial sciatic nerve ligation injury, partial infraorbital nerve ligation, chronic constriction of the infraorbital nerve injury and STZ-induced diabetes in mice. Therefore, PAF antagonists might be promising molecules for the treatment of chronic neuropathic pain. However, the possibility should always be considered that PAF antagonists might produce side effects by interfering with the physiological roles of PAF, such as regulation of the blood pressure, immunological or inflammatory responses, Ca^{2+} mobilization in polymorphonuclear leukocyte, or implantation of embryos, as shown by the creation of PAF receptor-transgenic and PAF receptor-deficient mice (Honda et al., 2002).

In a phase II clinical study in septic patients TCV-309, 1 mg/kg, twice daily, intravenously for 14 days has been shown to achieve a substantial reduction in organ dysfunction and morbidity, frequently associated with septic shock, and without significant adverse events, although it did not change the overall mortality due to septic shock (Froon et al., 1996; Poeze et al., 2000). In considering the clinical treatment of neuropathy by PAF antagonists, it is valuable that TCV-309 was effective against neuropathic pain caused by different pathologies, at low doses (ED_{50}: 1.5~10.8 μg/kg, i.v. and ED_{50}: 16~110 μg/kg, p.o.), and the effect was constant with repeated administration. Other antagonists also showed a similar profile of anti-allodynia effects. It is particularly remarkable that the anti-allodynia action of PAF antagonists lasted as long as 5–7 days by single i.v. injection of them comparing to the transient effect of gabapentin in partial sciatic nerve ligation injury model.

Amelioration of allodynia in nerve injury mice by i.t. injection of PAF antagonists and knockdown of spinal PAF receptors by PAF receptor siRNA suggests the involvement of a spinal action site for PAF antagonists. Inhibitory glycinergic neurons and GlyRs are abundant in the dorsal horn, where they play an important role in the prevention of pathological pain symptoms. Recent studies have emphasized that dysfunction of inhibitory neuronal regulation of pain signal transduction may be relevant to the development of neuropathic pain. Actually, significant disinhibition following alteration in glycine-mediated inhibition may occur after peripheral nerve injury (Dohi et al., 2009). Cyclic AMP-dependent protein

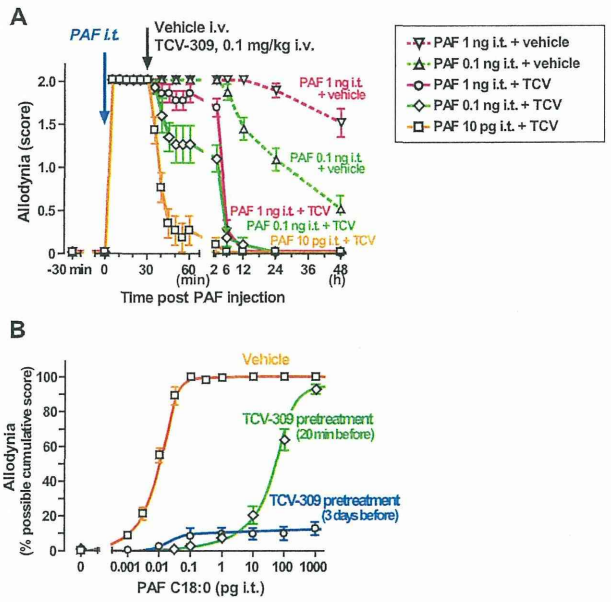

Figure 5 Mode of anti-allodynia action of TCV-309. (A) Time-course of anti-allodynia effects of intravenous injection of TCV-309 (0.1 mg/kg) 30 min after intrathecal injection with various doses of platelet-activating factor (PAF); (△; 0.1 ng + vehicle), (▽; 1 ng + vehicle), (◊; 0.1 ng + TCV-309), (○; 1 ng + TCV-309), (□; 10 ng + TCV-309) on tactile allodynia. Tactile allodynia was assessed by lightly stroking the flank of each mouse with a paintbrush on both sides of the leg. The score for each mouse was obtained from the average of score from both side trials. Values represent the allodynia score evaluated at each time point as the mean ± standard error of the mean (SEM) (n = 8–10 mice per group). (B) Time-dependent effects of intravenous injection of TCV-309 (0.1 mg/kg) on tactile allodynia induced by intrathecal injection with various doses of PAF. Tactile allodynia induced by PAF (0.01–1000 pg, i.t.) in mice treated with TCV-309 (0.3 mg/kg i.v.) or a vehicle at 30 min and 3 days after the injection. Each point represents the percent of the maximal possible cumulative score over 60 min evaluated every 5 min as the mean ± SEM (n = 7–13 mice per group). Control mice received injections with a vehicle: isotonic saline. Tactile allodynia induced by intrathecal injection with various doses of PAF in mice was not affected by vehicle treatment.

kinase phosphorylates and inhibits a specific subtype of GlyR, GlyRα3, which is distinctly expressed in the superficial dorsal horn and normally controls the excitability of neurons (Ahmadi et al., 2002; Gao and Ji, 2010). This mechanism mediates the central sensitization of inflammatory pain by prostaglandin E_2 through activation of EP_2 receptors and adenylate cyclase (Ahmadi et al., 2002; Rácz et al., 2005; Hösl et al., 2006; Gao and Ji, 2010). An increase in the NO/cyclic GMP cascade reduces GlyRα3 function in the spinal cord is involved in the hyperalgesia and allodynia induced by PAF (Morita et al., 2008a). TCV-309 at an adequate dose to produce anti-allodynia in partial sciatic nerve ligation mice failed to ameliorate the allodynia in mice transfected with GlyRα3 siRNA by siRNA for GlyRα3-mRNA in injured mice. This suggests that PAF antagonists may relieve PAF-induced reduction of GlyRα3-mediated inhibitory control of pain signal transduction in the spinal cord.

We have previously reported that GlyT inhibitors, by enhancing spinal glycinergic inhibition, produced an anti-allodynia action (Morita et al., 2008b) and thus proposed that GlyTs are a target for drug discovery for neuropathic pain (Dohi et al., 2009). However, GlyTs inhibitors lack an inhibitory effect on allodynia in the early stage of allodynia development after nerve injury (for 3 to 4 days after surgery), while they produced a long-lasting anti-allodynia action against established allodynia. On the contrary, knockdown of spinal GlyRα3 by GlyRα3 siRNA blocked the initiation of allodynia only during the first 3 days post surgery. This reversed effect of GlyT inhibition on neuropathic pain can be explained by the hypothesis that a reduction in the chloride gradient across the neuronal membrane, which in turn leads to reduction of the anion reversal potential, occurs in neurons of lamina I of the superficial dorsal horn following peripheral nerve injury (Coull et al., 2003), and the change in driving force means that glycine receptor-mediated input produces less hyperpolarization and could even paradoxically depolarize the neuron, while microglia and neuron interaction via brain-derived neurotrophic factor (Coull et al., 2005) may contribute to the phase-dependent anti-allodynia effect of GlyT inhibitors. According to these events, PAF antagonists, if their action is due solely to mediation by activation of GlyRα3, may also be expected to lose their inhibitory action in the early stage of allodynia induced by nerve injury. However, pretreatment with PAF antagonists or siRNA of PAF receptor mRNA before surgery inhibited the initiation of allodynia responses. This result suggests that the anti-allodynia effect of PAF antagonists in the early stage of allodynia development may be due to a different mechanism from relief from PAF-induced disinhibition of GlyRα3.

Another remarkable aspect of PAF antagonist-induced anti-allodynia action is its long-lasting effect. To clarify the mechanism behind the long action of PAF antagonists, the mode of action of TCV-309 against PAF-induced allodynia was investigated. *In vivo* analysis revealed a unique mode of action of TCV-309; the potency of TCV-309 intensified as a function of time after the administration, and the mode of action changed from a competitive manner in the early period after the injection of TCV-309 and to a non-competitive manner in the later period. TCV-309 is a specific competitive inhibitor of PAF receptors with no partial agonistic activity (Terashita et al., 1992) and

this explains the competitive mode of action in the early phase. Next, the intensification of the anti-allodynia potency of TCV-309 and change in its mode of action to a non-competitive manner led us to speculate about the different mechanism of action; such as down-regulation of PAF receptors by binding TCV-309 to PAF receptors. The decrease in surface expression of PAF receptors by TCV-309 in the plasma membrane of microglia isolated from the mouse spinal cord according to the incubation time with TCV-309 (unpublished observation) may support this idea. Further studies would be required to explain the long-lasting action of PAF antagonists by this speculation. To assess another possibility regarding whether PAF antagonists remain in the target tissue during their effective period, pharmacokinetic analysis of the compounds remains to be carried out.

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Author contributions

N.M., K.M., T.K. and T.D. oversaw the overall execution of the project, contributed to the experimental design and the interpretation of the results. S.S., Y.U., F.N. and T.K. performed the research. N.M., K.M. and T.K. analysed the data. N.M., K.M. and T.D. wrote the paper. All authors discussed the results and commented on the manuscript.

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