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Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice

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Abstract

Our previous study showed that intrathecal (i.t.) injection of platelet-activating factor (PAF) induced tactile allodynia, suggesting that spinal PAF is a mediator of neuropathic pain. The present study further examined the spinal molecules participating in PAF-induced tactile allodynia in mice. I.t. injection of L-arginine, NO donor (5-amino-3-morpholinyl-1,2,3-oxadiazolium (SIN-1) or 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-18)) or cGMP analog (8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate; pCPT-cGMP) induced tactile allodynia. PAF- and glutamate- but not SIN-1- or pCPT-cGMP-induced tactile allodynia was blocked by an NO synthase inhibitor. NO scavengers and guanylate cyclase inhibitors protected mice against the induction of allodynia by PAF, glutamate and SIN-1, but not by pCPT-cGMP. cGMP-dependent protein kinase (PKG) inhibitors blocked the allodynia induced by PAF, glutamate, SIN-1 and pCPT-cGMP. To identify signalling molecules through which PKG induces allodynia, glycine receptor $\alpha 3$ (GlyR $\alpha 3$) was knocked down by spinal transfection of siRNA for GlyR $\alpha 3$. A significant reduction of GlyR $\alpha 3$ expression in the spinal superficial layers of mice treated with GlyR $\alpha 3$ siRNA was confirmed by immunohistochemical and Western blotting analyses.

Functional targeting of GlyR $\alpha 3$ was suggested by the loss of PGE₂-induced thermal hyperalgesia and the enhancement of allodynia induced by bicuculline, a GABA_A receptor antagonist in mice after GlyR $\alpha 3$ siRNA treatment. pCPT-cGMP, PAF, glutamate and SIN-1 all failed to induce allodynia after the knockdown of GlyR $\alpha 3$. These results suggest that the glutamate-NO-cGMP-PKG pathway in the spinal cord may be involved in the mechanism of PAF-induced tactile allodynia, and GlyR $\alpha 3$ could be a target molecule through which PKG induces allodynia.

1. Introduction

Platelet-activating factor (PAF) is a lipid mediator of the inflammatory response. PAF is elevated in cerebrospinal fluid in association with various diseases accompanied by abnormal pain sensation, such as spinal cord injury [32] and relapsing-remitting multiple sclerosis [4], and contributes to robust inflammatory responses in the acute phase and secondary injury in spinal cord injury [13, 23] and in experimental allergic encephalomyelitis [29]. Although PAF is believed to be a potent edematous mediator, but not a pain mediator, in peripheral tissues, we recently demonstrated that intrathecal injection of PAF produced potent tactile allodynia and thermal hyperalgesia in mice [37]. Thus, PAF may be a mediator of noxious signalling in the spinal cord in some cases of neuronal injury.

PAF-induced tactile allodynia may be mediated by ATP, glutamate and the generation of nitric oxide (NO) [37]. A body of evidence suggests that the activation of NMDA receptors and subsequent production of NO and cGMP are involved in persistent pain and tactile allodynia in chronic inflammatory and nerve-injury models [11, 12, 15, 31, 45, 46, 55]. However, several lines of evidence suggest that the NO/cGMP signalling cascade has either pro- or anti-allodynia effects in various

neuropathic pain models [18, 20, 41, 50], as well as discordant effects in peripheral inflammatory models. The differing effects of the NO/cGMP signal cascade concerning noxious transduction may be due at least in part to differences in experimental conditions under which neuronal background activity, including the activity of the NMDA/NO/cGMP system, was examined [20].

Accumulating evidence supports the notion that the generation of pain is due to specific dysfunction of the glycinergic inhibitory system in the spinal cord [56]. Such dysfunction may be due to altered glycine receptor (GlyR) function, causing depolarization of spinal cells in response to the appearance of neurotransmitter glycine in peripheral nerve injuries [7, 8, 43]. PGE₂ leads to inhibition of the function of GlyR containing the $\alpha 3$ subunit (GlyR $\alpha 3$) in the superficial dorsal horn. This inhibition is relevant to PGE₂-evoked pain [2, 19]. The objective of this study was to elucidate whether elevation of endogenous NO and the resultant activation of the cGMP/protein kinase G (PKG) pathway constitute the signalling pathway for PAF- and glutamate-induced tactile allodynia by using specific agents affecting the signalling pathway. To further identify the target molecule for PKG mediation of tactile allodynia, the involvement of GlyR in the spinal cord was investigated. For this purpose, the knockdown effect of spinal GlyR $\alpha 3$ subunit by siRNA for GlyR $\alpha 3$ on the induction of

tactile allodynia by cGMP analog and the PAF-glutamate-NO cascade was examined.

2. Materials and methods

2.1. Animals

Male ddY mice (Kyudo, Kumamoto, Japan) weighing 25–30 g were used. All procedures and handling of animals were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society as well as the guidelines of Hiroshima University, Hiroshima, Japan.

2.2. Injection

For intrathecal (i.t.) injection, the head of a mouse was placed into a plastic cap and the body was held with one hand as described previously [14]. A 27-gauge needle attached to a Hamilton microsyringe was inserted into the subarachnoid space between the L5 and L6 vertebrae of the conscious mouse and 5 μ l of drug solution was slowly

injected, as described by Hylden and Wilcox [24]. Accurate placement of the needle was confirmed by a quick “flick” of the mouse’s tail, as described by Honda et al. [21].

2.3. General testing procedures

For the measurement of touch-evoked tactile allodynia, each mouse was placed in an individual plastic cage. Tactile allodynia was assessed by lightly stroking both sides of the flank of the mice with a paintbrush. Brushing was performed twice on each side from a backward direction on the lower half of the left and right sides of the flank of the mouse. Tactile allodynia response was ranked as described by Minami et al. [34]: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; 2, vigorous squeaking, biting the stroking probe and strong efforts to escape from the stroking probe. The score for each mouse was obtained from the average of scores from both side trials. In some experiments, tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments (von Frey hairs). The animals were placed in a plastic cage. After allowing the animals to adapt to the environment, numbered von Frey monofilaments were applied perpendicularly to the dorsal surface of the left and right hind paws. The

smallest filament that caused the animal to flinch or move the paw away from the stimulus three times out of three trials was determined to be the mechanical threshold. The filament number was converted to buckling force (gm). The value for each mouse was obtained from the average buckling force of both hind paws. For the measurement of thermal hyperalgesia, mice were placed on a 52 ± 0.1 °C hot plate. The response latency to either a hind-paw lick or jump was recorded. Injection and observation of behavior were performed by blind different observers.

2.4. Analysis with small interfering RNA (siRNA) against glycine receptor $\alpha 3$ (GlyR $\alpha 3$)

We selected three target sequences consisting of nucleotides 1042–1066 (sense GlyR $\alpha 3$ _siRNA#1), 683–707 (sense GlyR $\alpha 3$ _siRNA#2) and 1356–1380 (sense GlyR $\alpha 3$ _siRNA#3) downstream of the start codon of GlyR $\alpha 3$ mRNA: sense siRNA#1, 5'-AGGUUUCGGCGAAAGAGAAAGAAUA-AG-3'; sense siRNA#2, 5'-GGUACUGCACUAAACACUACAAUAC-AG-3'; and sense siRNA#3, 5'-CCUUAGGCAUGAAGACAUUCAUCAU-AG-3'. Moreover, mismatched siRNA with three nucleotide mismatches was prepared to examine nonspecific effects of siRNA duplexes (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 (GlyR $\alpha 3$) in the mouse genome was targeted. siRNAs were purchased from iGENE Therapeutics Inc. (Tsukuba, Japan).

For *in vivo* siRNA transfer, the hemagglutinating virus of the Japan envelope vector system (HVJ Envelope Vector Kit GenomeONE; Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) was used. This HVJ Envelope (HVJ-E) vector has been proven to be an effective oligodeoxynucleotide (ODN) delivery system both *in vitro* and *in vivo* [28]. siRNAs were incorporated into HVJ-E vector according to the manufacturer's instructions. Briefly, after 40 μ l (1 assay unit, AU) of HVJ-E vector was mixed with 4 μ l of the enclosing factor, the mixture was centrifuged (10,000 \times g, 10 min, 4 $^{\circ}$ C), and the pellet was suspended in 10 μ l of the buffer solution. Then, 10 μ l of a mixture of 3 siRNAs (#1, #2 and #3, 1 μ g/ μ l each) was added, and the mixture was kept on ice for 5 min. Sterile artificial cerebrospinal fluid (ACSF, 5 μ l) containing synthetic siRNA duplexes (0.45 pmol/animal) was injected into the subarachnoid space between the L5 and L6 vertebrae of conscious mice. At the times indicated in the text, mice were anesthetized with pentobarbital (65 mg/kg i.p.), and the spinal cords were subjected to immunoblotting and immunohistochemical analysis. For the controls, we followed the

same procedures; however, we substituted an identical amount of mismatched siRNA#4 with HVJ-E vector and only HVJ-E vector without siRNA.

2.5. Immunohistochemical analysis

Untreated animals or animals treated with HVJ-E, GlyR siRNA or mutant siRNA were anesthetized with pentobarbital, followed by transcardial perfusion with 0.9 % saline and then with 4 % paraformaldehyde (PA) in 0.1 M phosphate buffer (PB) (pH 7.4). The lumbar regions of the spinal cord (L2–L6) were removed and post-fixed for 2 hr with 4 % PA, followed by cryoprotection with 30 % sucrose in PB at 4 °C for 2 days. Coronal sections were cut at 10- μ m thickness with a cryostat at -24 °C. The sections were incubated with primary antibody against GlyR α 3 (1:50; developed in rabbits, affinity isolated polyclonal antibody, SIGMA Aldrich Inc., St. Louis, MO, USA) at 4 °C overnight. After three washes in PBS, the sections were reacted at room temperature for 1 hr with a biotinylated secondary antibody (1 : 200; BA-1300 Vector Laboratories, Burlingame, CA, USA). After rinsing for 5 min in PBS, the sections were placed in solutions containing avidin and biotinylated peroxidase at room temperature for 1 hr, according to the manufacturer's instructions. The nonspecific signal was not calibrated

due to the lack of an available standard for GlyR $\alpha 3$ protein and a blocking peptide with which to verify antibody specificity.

2.6. Western blotting

2.6.1. Samples

Lumbar regions of the spinal cord (L2–L6) were obtained from untreated animals or animals injected with HVJ-E, GlyR siRNA or mutant siRNA. The spinal cord tissues (4.8–5.0 mg) were homogenized in homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 10 % (v/v) glycerol, 10 mM NaF, 10 mM β -glycerophosphate and 1 μ g/ml of various protease inhibitors (benzamidine, leupeptin and antipain). The homogenate was incubated with 0.6 % NP-40 for 5 min at 4 °C, and then centrifuged at 20,000 x g for 5 min at 4 °C, and the supernatant was collected. The supernatant was mixed at a volume ratio of 4 : 1 in 10 mM Tris-HCl (pH 6.8) containing 10 % (v/v) glycerol, 2% sodium dodecylsulfate (SDS), 0.01 % bromophenol blue and 0.5 % β -mercaptoethanol, followed by boiling at 100 °C for 10 min.

2.6.2. Immunoblotting assays

The samples (30 μ g of crude protein extracts) were applied to polyacrylamide gels (stacking gel 3 % and separating gel 10 %), electrophoresed for 2 hr at room temperature and then blotted onto polyvinylidene fluoride membranes previously treated with 100 % methanol. The transferred proteins on the membranes were blocked by incubating with blocking solution, i.e., washing buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 0.1 % Tween 20) containing 5 % skim milk at 25 °C for 1 hr. The membranes were exposed to the primary antibody against GlyR α 3 (1 : 500; SIGMA Aldrich Inc., St Louis, MO, USA) diluted in washing buffer containing 1 % skim milk overnight at 4 °C, washed three times with washing buffer, and then incubated with a biotinylated secondary antibody (1 : 1,000) at 4 °C for 1 hr. After rinsing for 5 min in washing buffer, membranes were placed in a solution containing avidin and biotinylated peroxidase at 25 °C for 1 hr. The membranes were again rinsed three times with washing buffer and developed with ECLTM, followed by exposure to X-ray films. Densitometry was carried out on these X-ray films with the aid of an ATTO Densitograph 4.0 (Atto Corporation, Tokyo, Japan).

2.7. Experimental protocols

The names of drugs used, their respective mechanisms of action, route of administration and references are summarized in Table 1.

The first experiment examined whether several NO donors and an analogue of cGMP evoked tactile allodynia in the present experimental model. On a given test day, a group of mice received i.t. injection of 5 μ l of either L-arginine, SIN-1, NOC-18, pCPT-cGMP or ACSF (drug vehicle). Tactile allodynia was assessed before and after the i.t. injection.

A separate group of mice was used in a second experiment to examine the influence of 7-NINA, TRIM, hemoglobin, carboxy-PTIO, ODQ, NS 2028, Rp-8-pCPT-cGMPS or KT 5823 on the induction of tactile allodynia by PAF, glutamate, SIN-1 or pCPT-cGMP. On a given test day, 7-NINA or TRIM was intrathecally injected 20 min before i.t. injection of 5 μ l of either PAF, glutamate, SIN-1, pCPT-cGMP or ACSF. Hemoglobin, carboxy-PTIO, ODQ, NS 2028, Rp-8-pCPT-cGMPS or KT 5823 was injected intrathecally 20, 20, 60, 300, 30 and 30 min before the i.t. injection of PAF, glutamate, SIN-1 or pCPT-cGMP. Doses of 0.19 fmol (i.t.) and 5.0 pmol (i.t.) for PAF and glutamate, respectively, were used, as in previous studies [14, 37]. Tactile allodynia was assessed before and after the injection.

Another group of mice received spinal transfer of siRNA against GlyR α 3 to

examine the involvement of spinal GlyR $\alpha 3$ in pCPT-cGMP-, PAF-, glutamate- and SIN-1-induced tactile allodynia. Knockdown of GlyR $\alpha 3$ protein after spinal transfection of GlyR $\alpha 3$ was confirmed by immunohistochemical analysis. Three days after the knockdown of GlyR $\alpha 3$ by siRNA, tactile allodynia responses to PAF, glutamate, SIN-1 and pCPT-cGMP were examined. The knockdown effect of GlyR $\alpha 3$ on allodynia induced by bicuculline, a GABA_A receptor antagonist, was also examined.

2.8. Preparation of drug solution

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 ACSF. SIN-1, NOC-18, pCPT-cGMP, glutamate, Rp-8-pCPT-cGMPS, carboxy-PTIO and hemoglobin were dissolved in ACSF. 7-NINA, TRIM and KT 5823 were dissolved in 0.1 % dimethyl sulfoxide (DMSO). ODQ and NS 2028 were dissolved in 0.4 % DMSO.

2.9. Data analysis

Values are presented as a percentage of the possible maximal score evaluated at each time point (possible maximum score at each time point: 2/mouse) 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 x 12 trials = 24), as described previously [37]. The area under the allodynia scores and withdrawal threshold against the time curve (AUC) were calculated by the trapezoidal method. Regarding the statistical analysis of tactile allodynia, comparisons of allodynia scores of the vehicle versus various inhibitors or scavengers (Figs. 2, 3), and comparisons of the AUC of allodynia scores and withdrawal threshold differences between treatments (Figs. 4 to 6) were analyzed by one-way ANOVA followed by Tukey's test. Statistical analyses of comparisons of allodynia scores and the withdrawal threshold of the corresponding control just prior to versus after the injection of drugs (Figs. 4 to 6) were performed using a paired *t*-test. Statistical analysis of Western blotting analysis was performed with Fisher's PLSD. A *P* value less than 0.05 was regarded as significant.

3. Results

3.1. Tactile allodynia induced by precursors and NO donors of NO and cGMP analogue

The induction of tactile allodynia by i.t. injection of a precursor or donors of NO and a cGMP analogue in normal mice was examined. L-arginine (136 nmol) (NO precursor) but not D-arginine (136 nmol), and SIN-1 (48.4 nmol) and NOC-18 (61.3 nmol) (NO donors) produced allodynia in response to innocuous stimuli applied to the flank shortly after injection, and the response lasted for 60 min (Fig. 1A). I.t. injection of pCPT-cGMP, a membrane-permeable and phosphodiesterase-resistant analog of cGMP, also produced marked, long-lasting tactile allodynia over the concentration range of 0.39 pmol to 0.97 nmol, with maximal effect at 0.29 nmol/mouse (Fig. 1B, C). Doses of 48.4 nmol for SIN-1 and 0.29 nmol for pCPT-cGMP were used in the following experiments.

3.2. The effects of NOS inhibitor and NO scavengers on tactile allodynia induced by PAF, glutamate, SIN-1 and pCPT-cGMP

The effects of NOS inhibitors 7-NINA and TRIM, and NO scavengers, hemoglobin and carboxy-PTIO, on tactile allodynia induced by PAF, glutamate, SIN-1 and

pCPT-cGMP were examined. The doses of the inhibitors causing maximum inhibition of PAF-induced tactile allodynia were used in the following experiments. Pretreatment with 7-NINA or TRIM (NOS inhibitors) dose-dependently blocked PAF- and glutamate-induced tactile allodynia, but these inhibitors, even at doses that produced the maximal effect against PAF or glutamate, did not block the allodynia response to SIN-1 and pCPT-cGMP (Fig. 2A). Pretreatment with hemoglobin and carboxy-PTIO (NO scavengers) blocked the induction of tactile allodynia by PAF, glutamate and SIN-1 but did not block the allodynia response to pCPT-cGMP at doses that produced the maximal effect against PAF, glutamate or SIN-1 (Fig. 2B).

3.3. The effects of G-cyclase inhibitors and PKG inhibitor on tactile allodynia induced by PAF, glutamate, SIN-1 and pCPT-cGMP

Pretreatment with ODQ and NS 2028 (G-cyclase inhibitors) blocked the induction of tactile allodynia by PAF, glutamate and SIN-1, but not by pCPT-cGMP at doses that produced the maximal effect against PAF, glutamate or SIN-1 (Fig. 2C). Pretreatment with Rp-8-pCPT-cGMPS (a PKG inhibitor) blocked the induction of tactile allodynia by pCPT-cGMP in a dose-dependent (Fig. 2D) and time-dependent (Fig. 2E) manner,

with the maximal effect at 9.1 nmol (Fig. 2D). Rp-8-pCPT-cGMPS and KT 5823 (PKG inhibitors) blocked PAF-induced allodynia with a similar dose-response relationship as that against pCPT-cGMP-induced allodynia (Fig. 2D). Allodynia induced by PAF, glutamate and SIN-1 was blocked by 9.1 nmol Rp-8-pCPT-cGMPS (Fig. 2D) at all times examined (Fig. 2F).

3.4. Knockdown of spinal GlyR $\alpha 3$ subunit by siRNA

To explore the target molecule for PKG associated with the induction of tactile allodynia, mice with knockdown of the expression of GlyR $\alpha 3$ subunit protein were prepared by intrathecal transfer of GlyR $\alpha 3$ siRNA. Immunoreactive signals of GlyR $\alpha 3$ subunit protein antibody were detected in the superficial spinal layers of mice (Fig. 3Aa), but such signals were not detected in control staining without an antibody (Fig. 3Ab). Marked reduction of GlyR $\alpha 3$ expression in the superficial spinal layers of mice treated with GlyR $\alpha 3$ siRNA (Fig. 3Ac) compared with untreated, HVJ-E vector-injected or mutant-siRNA-injected control mice (Fig. 3Ac, e) was detected 3 days after treatment by immunohistochemical analysis, but the reduction had ceased by 10 days after treatment with GlyR $\alpha 3$ siRNA (Fig. 3Af-h). Western blotting analysis

showed a time-dependent reduction from 1 to 5 days after treatment with GlyR $\alpha 3$ siRNA but not mutant siRNA, with the most marked reduction at 3 days after injection (Fig. 3B, C). In the absence of an antibody, no band corresponding to GlyR $\alpha 3$ was observed (Fig. 3B). No apparent changes in spontaneous behavior assessed by posture, gait, activity of spontaneous movement and defecation were observed during this period. The following experiments for evaluation of the effect of GlyR $\alpha 3$ knockdown were performed 3 days after treatment with GlyR $\alpha 3$ siRNA. The effect of introducing GlyR $\alpha 3$ siRNA into the spinal cord on PGE₂-induced thermal hyperalgesia was examined to verify that the present method interfered with PGE₂-induced noxious hypersensitization. The threshold of thermal hyperalgesia by the hot plate test was slightly reduced in mice 3 days after treatment with GlyR $\alpha 3$ siRNA compared to normal mice (Fig. 3D). PGE₂ significantly reduced the threshold of thermal hyperalgesia in vehicle-treated mice, and this reduction was not observed in mice treated with GlyR $\alpha 3$ siRNA. PAF-induced sensitization of thermal hyperalgesia also disappeared in mice treated with GlyR $\alpha 3$ siRNA (Fig. 3E).

3.5 Effects of GlyR $\alpha 3$ knockdown on bicuculline-induced tactile allodynia

To verify that the transfection of GlyR $\alpha 3$ siRNA did not interfere nonspecifically with inhibitory control by the GABAergic system, the effect of GlyR $\alpha 3$ siRNA treatment on the induction of allodynia by bicuculline, a GABA_A receptor antagonist, was examined. The injection of ACSF in mice treated with HVJ-E, mutant siRNA and GlyR $\alpha 3$ siRNA produced only small and transient changes in the allodynia score and in the paw withdrawal threshold (Fig. 4A–C). Bicuculline induced allodynia at 0.27 (i.t., minimal dose) to 2.7 nmol (i.t., submaximal dose), and induced convulsions at more than 27 nmol (i.t.). The scores of allodynia induced by bicuculline (2.7 nmol, i.t.) in HVJ-E- or mutant siRNA-injected control mice (Fig. 4D, E) were not reduced but rather increased in siRNA-treated mice (Fig. 4F), and the duration of allodynia was prolonged from 3.5 hrs in the control (Fig. 4D, E) to 10 hrs in siRNA-treated mice (Fig. 4F). The withdrawal threshold assessed by the von Frey hair test was also markedly decreased and prolonged in siRNA-treated mice (Fig. 4D–F). AUC of the allodynia score and withdrawal threshold in GlyR $\alpha 3$ siRNA-treated mice significantly increased compared to those in HVJ-E or mutant siRNA-treated mice (Fig. 4D–F).

3.6. GlyR $\alpha 3$ subunit as a target molecule for glutamate-NO-cGMP system

It was examined whether GlyR $\alpha 3$ is involved in the induction of tactile allodynia induced by PAF and the downstream cascade in GlyR $\alpha 3$ siRNA-treated mice. I.t. injection of pCPT-cGMP failed to induce tactile allodynia as assessed by either the paintbrush test or von Frey hair test in GlyR $\alpha 3$ siRNA-treated mice (Fig. 5C), while it produced tactile allodynia in control mice injected with HVJ-E (Fig. 5A) or mutant GlyR $\alpha 3$ siRNA (Fig. 5B). On the 12th day after injection of GlyR $\alpha 3$ siRNA (after the expression of GlyR $\alpha 3$ protein was restored), the ability of pCPT-cGMP to induce allodynia was recovered (Fig. 5D). The AUC of the allodynia score and withdrawal threshold 3 days after injection of HVJ-E, mutant siRNA and 12 days after injection of GlyR $\alpha 3$ siRNA were not significantly different, whereas those values were markedly decreased 3 days after injection of GlyR $\alpha 3$ siRNA (Fig. 5A–D). PAF, glutamate and SIN-1 also failed to induce tactile allodynia in mice pretreated with GlyR $\alpha 3$ siRNA (Fig. 6B, D, F), while tactile allodynia was induced by these agents in HVJ-E- or mutant siRNA-injected control mice (Fig. 6A, C, E).

4. Discussion

ATP P2X and NMDA receptor activation have been shown to be involved in PAF-induced tactile allodynia [37]. The present results demonstrating the blockade of PAF-induced tactile allodynia by NOS inhibitors (7-NINA and TRIM) and by NO scavengers (hemoglobin and carboxy-PTIO), and the ability of L-arginine, SIN-1 or NOC-18 (precursor or donors for NO) to produce tactile allodynia in normal mice suggest that NO formed endogenously following PAF receptor stimulation may contribute to PAF-induced allodynia. Among various target molecules for NO, the activation of soluble G-cyclase and the subsequent stimulation of cGMP/PKG is one of the major pathways by which NO produces biological events [39]. The blockade by G-cyclase inhibitors (ODQ and NS 2028) of glutamate- and NO-supplying agent-induced tactile allodynia suggests that G-cyclase/cGMP are downstream effectors of signalling for the induction of tactile allodynia by the glutamate/NO system. In addition, pCPT-cGMP (a cGMP analogue) had potent ability to induce tactile allodynia upon i.t. injection. The antagonism by PKG inhibitor but not by NOS inhibitor, NO scavengers or G-cyclase inhibitor of pCPT-cGMP-induced tactile allodynia suggests that the allodynic effect of pCPT-cGMP was not due to its indirect effects on ion

channels via its amphipathic property but rather due to the activation of PKG. The specificity of inhibitors is a point that must be considered in any pharmacological study. We confirmed that two different inhibitors produced similar effects: NOS inhibitors blocked the response to glutamate but not to direct NO donors and the downstream pCPT-cGMP, whereas NO scavengers and G-cyclase inhibitors blocked the response to NO donors but not to downstream pCPT-cGMP even at doses that produced maximal effects against the response to glutamate. These results are in agreement with the evidence that the activation of cGMP-dependent protein kinase 1 α is required for NMDA- or NO-induced spinal thermal hyperalgesia [53].

PAF receptors couple with a variety of intracellular signalling mediators [27]. We have previously reported that PAF inhibits Na⁺,K⁺-ATPase activity in adrenal chromaffin cells through mitogen-activated protein kinase [10] and that the inhibition of Na⁺,K⁺-ATPase activity increases intracellular Na⁺ and Ca²⁺ concentration [36]. These events result in stimulation of the secretory response in adrenal chromaffin cells [38]. Taken together with the fact that PAF receptor mRNA is expressed in DRG [37], these findings suggest the possibility that PAF stimulates glutamate release from primary afferent neurons in the spinal cord by a similar mechanism. The present results of step-by-step blockade by NOS inhibitor, NO scavengers, G-cyclase inhibitors and a

PKG inhibitor of PAF-, glutamate-, NO donors- and cGMP analog-induced allodynia suggest that an important cascade by which PAF induces tactile allodynia may consist of glutamate/NMDA receptor interaction followed by NO/G-cyclase and then cGMP/PKG signalling.

The target molecules for PKG remain to be identified in future studies. Ion channels in nociceptors are expected to be one of the target molecules for the modulation of nociception by cGMP. The cGMP/PKG and cAMP/PKA pathways play roles in maintaining the neuronal hyperactivity of DRG neurons, as shown by decreases in the action potential threshold and increased repetitive discharge during depolarization and behavioral hyperalgesia in a neuropathic pain model involving chronic compression of the DRG and modulation using agonists and antagonists for PKG and PKA, [49]. The majority of inhibitory neuronal systems in the spinal cord contain both glycine receptors and GABA_A receptors, which consist of Cl⁻ channels [42, 51, 54]. Reduction of the transmembrane chloride gradient in spinal lamina I neurons contributes to the cellular hyperexcitability producing allodynia and hyperalgesia after nerve injury [7, 8, 43, 56]. Thus, relief from the inhibition of dorsal horn neurons due to dysfunction of these inhibitory systems contributes to sensitization to pain signals. Actually, the inhibition of glycine and GABA_A receptors induces tactile allodynia, and their

activation has antiallodynic effects [1, 9, 17, 33, 56].

In the adult dorsal horn, GlyR $\alpha 3$ is specifically expressed in the superficial laminae of the dorsal horn and contributes to dominant fast inhibitory postsynaptic transmission by glycine in the superficial layers [2, 19]. Harvey et al. [19] demonstrated that mice deficient in GlyR $\alpha 3$ lacked the inhibition of post-synaptic glycine current induced by PGE₂/EP₂-dependent activation of cAMP/PKA seen in wild-type mice, and showed a reduction in tactile or thermal hyperalgesia induced by spinal PGE₂ injection and in pain sensitization induced by peripheral inflammation. This suggests that the disinhibition of dorsal horn neurons by blocking GlyR $\alpha 3$ via PGE₂-activated protein kinase A (PKA)-dependent phosphorylation of the subunit is relevant to PGE₂-evoked tactile, thermal and inflammatory pain sensation. Thus, GlyR $\alpha 3$ is suggested to be an essential target molecule for spinal PGE₂ through PKA-mediated pain sensitization. However, GlyR $\alpha 3$ does not contribute to acetic acid- and formalin-induced inflammatory pain or sciatic nerve ligature-induced neuropathic pain [22, 44]. Here we examined whether GlyR $\alpha 3$ is a target molecule for the induction of tactile allodynia by PKG. For this purpose, the expression of GlyR $\alpha 3$ was suppressed by i.t. injection of siRNA of GlyR $\alpha 3$. It was recently reported that synthetic RNA duplexes 25–30 nucleotides in length can be more potent and have a longer duration of action than

corresponding conventional 21-mer small siRNA *in vitro* [30, 47]. We used a mixture of 3 duplex 27-nucleotide siRNAs for silencing GlyR $\alpha 3$. The present results demonstrated that the transfection of siRNA of GlyR $\alpha 3$ using HVJ-E effectively suppressed immunoreactive signals against GlyR $\alpha 3$ antibody. Although the possibility of nonspecific binding of the antibody was not completely excluded, the specific reduction of the signal in the superficial laminae of the dorsal horn by the introduction of GlyR $\alpha 3$ siRNA but not mutant siRNA may indicate selective reduction of GlyR $\alpha 3$ protein by siRNA.

Western-blotting analysis revealed the maximum reduction of the protein expression (by about 75 %) at 3 days post-transfection, and PGE₂-induced thermal hyperalgesia was counteracted by knockdown of GlyR $\alpha 3$ using its siRNA. These data are consistent with the phenotype of GlyR $\alpha 3$ knockout mice [19]. PAF-induced thermal hyperalgesia also disappeared in GlyR $\alpha 3$ knockdown mice. These results clearly demonstrated the effectiveness of the introduction of GlyR $\alpha 3$ siRNA into the spinal cord for interfering with the function of GlyR $\alpha 3$ *in vivo*.

GABA_A receptors are distributed throughout the dorsal horn and contribute to the regulation of noxious signal transduction. Thus, it needs to be verified that the function of GABA_A receptors was not disturbed by the transfer of GlyR $\alpha 3$ siRNA. Actually,

tactile allodynia induced by GABA_A antagonist bicuculline was not blocked but rather markedly enhanced in mice with GlyR $\alpha 3$ knockdown. The increased response to bicuculline may reflect the reduction of glycinergic inhibitory control. This suggests that specific functional reduction of GlyR $\alpha 3$ was achieved without disrupting GABA_A receptor function using the present method. A cGMP analog (pCPT-cGMP) lost its ability to induce tactile allodynia in mice with GlyR $\alpha 3$ knockdown, but this ability was regained after immunoreactive GlyR $\alpha 3$ was recovered. These results suggest that the disinhibition of glycinergic inhibition by targeting GlyR $\alpha 3$ by PKG may be a cause of cGMP-induced allodynia. Whether PKG phosphorylates GlyR $\alpha 3$ or an associated protein and actually interferes with the receptor function remains an important issue to be clarified in further studies. To verify that a cascade involving PAF and glutamate/NMDA followed by NO/G-cyclase and then cGMP/PKG finally targets GlyR $\alpha 3$ to induce tactile allodynia, the effects of PAF and intermediates of the cascade were examined, and it was found that all PAF, glutamate and NO donors failed to induce tactile allodynia in GlyR $\alpha 3$ knocked-down mice. Thus, a cascade involving PAF and glutamate/NO may integrate with GlyR $\alpha 3$ in the spinal cord as a target molecule for the induction of allodynia. Taken together, the results of the present study suggest that a glutamate/NO and cGMP/PKG cascade by targeting GlyR $\alpha 3$ by PKG may be involved

in the induction of tactile allodynia by PAF.

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Figure legends

Figure 1. Time course of allodynia induced by intrathecal injection of NO donors, L-arginine (136 nmol; 5 μ l of 27.2 mM/mouse, i.t.), D-arginine (136 nmol; 5 μ l of 27.2 mM/mouse, i.t.), SIN-1 (48.4 nmol; 5 μ l of 9.7 mM/mouse, i.t.) and NOC-18 (61.3 nmol; 5 μ l of 12.3 mM/mouse, i.t.) (A), and a membrane-permeable, phosphodiesterase-stable analog of cGMP, pCPT-cGMP (1.0 nmol; 5 μ l of 200 μ M/mouse, i.t.) (B) in mice. Values represent allodynia scores assessed by the paintbrush test evaluated at each time point (mean \pm S.E., N=7-20). Dose-response relationship of pCPT-cGMP-induced tactile allodynia in mice (C). Values represent % maximal possible cumulative score over 60 min evaluated every 5 min (mean \pm S.E., N=7-13).

Figure 2. Effects of NOS inhibitors, 7-NINA and TRIM (A), NO scavengers, hemoglobin and carboxy-PTIO (PTIO) (B), G-cyclase inhibitors, ODQ and NS 2028 (C) and PKG inhibitor, Rp-8-pCPT-cGMPS (Rp) and KT 5823 (D) on tactile allodynia induced by PAF, glutamate, SIN-1 or pCPT-cGMP. 7-NINA (0.05, 0.15 and 0.50 nmol; 5 μ l of 10, 30 and 100 μ M/mouse, i.t.) and its vehicle (0.1 % DMSO), TRIM (0.3, 1.0

and 3.0 nmol; 5 μ l of 60, 200 and 600 μ M/mouse, i.t.) and its vehicle (0.1 % DMSO), hemoglobin (0.15, 0.45 and 1.50 nmol; 5 μ l of 30, 90 and 300 μ M/mouse, i.t.), carboxy-PTIO (PTIO; 3.2, 16.0 and 48.0 nmol; 5 μ l of 0.64, 3.2 and 9.6 mM/mouse, i.t.) or their vehicle (ACSF) was injected 20 min prior to i.t. injection of PAF (0.19 fmol; 5 μ l of 38 pM/mouse, i.t.), glutamate (5.0 pmol; 5 μ l of 1.0 μ M/mouse, i.t.), SIN-1 (48.4 nmol; 5 μ l of 9.7 mM/mouse, i.t.) or pCPT-cGMP (0.29 nmol; 5 μ l of 58.0 μ M/mouse, i.t.). ODQ (0.11, 1.1 and 11.0 nmol; 5 μ l of 0.022, 0.22 and 2.2 mM/mouse, i.t.) and its vehicle (0.4 % DMSO), NS 2028 (0.074, 0.74 and 7.4 nmol; 5 μ l of 0.015, 0.15 and 1.5 mM/mouse, i.t.) and its vehicle (0.4 % DMSO), Rp-8-pCPT-cGMPS (Rp; 1.7, 5.0 and 9.1 nmol; 5 μ l of 0.33, 1.0 and 1.8 mM/mouse, i.t.) and its vehicle (ACSF) or KT 5823 (2, 6 and 20 pmol; 5 μ l of 0.4, 1.2 and 4.0 μ M/mouse, i.t.) and its vehicle (0.1 % DMSO) were injected at 60, 300, 30 or 30 min prior to injection of PAF, glutamate, SIN-1 or pCPT-cGMP, respectively. Values represent % maximal possible cumulative score over 60 min evaluated every 5 min (mean \pm S.E., N=7-12). * P <0.01 compared with the corresponding control, as determined by analysis of variance followed by Tukey's test.

Figure 3. Effects of transfection of siRNA for GlyR α 3 on the expression of GlyR α 3.

A: Immunohistochemical analysis of spinal GlyR $\alpha 3$ after transfection of siRNA for GlyR $\alpha 3$ into spinal cord of mice. As controls, HVJ-E or mutant siRNA was injected into the spinal cord. The animals were perfused with 4 % paraformaldehyde under anesthesia at 3 or 10 days after i.t. administration. Spinal sections were stained with an antibody against GlyR $\alpha 3$. Scale bar represents 200 μm . B and C: Western blotting analysis of GlyR $\alpha 3$ after transfection of siRNA for GlyR $\alpha 3$, HVJ-E or mutant siRNA for GlyR $\alpha 3$ into the spinal cord of mice. The levels of GlyR $\alpha 3$ were normalized to β -actin and represented as % induction compared with the values of uninjured mice. Values are presented as % of naïve control (mean \pm S.E., N=4). * P <0.05 compared with the corresponding control, as determined by analysis of variance followed by Fisher's PLSD. D and E: Effect of GlyR $\alpha 3$ siRNA treatment on PGE₂- and PAF-induced thermal hyperalgesia. Experiments were performed 3 days after treatment of GlyR $\alpha 3$ siRNA. Thermal hyperalgesia was evaluated 10 min after injection of PGE₂ (5 nmol; 5 μl of 1 mM/mouse, i.t.) and PAF (0.19 fmol; 5 μl of 38 pM/mouse, i.t.). Values represent the response time of hind-paw lick or jump (mean \pm S.E., N=18). * P <0.01 compared with the corresponding control, as determined by analysis of variance followed by Tukey's test.

Figure 4. Effect of transfection of siRNA for GlyR $\alpha 3$ on tactile allodynia induced by bicuculline. A, B and C: Effect of i.t. injection of ACSF 3 days after the injection of GlyR $\alpha 3$ siRNA (C), mutant siRNA (B) or HVJ-E (A). Values represent the allodynia score assessed by the paintbrush test evaluated at each time point (\circ , \blacksquare) and the withdrawal threshold assessed by the von Frey hair test (\diamond). D, E and F: Effects of treatment with HVJ-E (D), mutant siRNA (E) or GlyR $\alpha 3$ siRNA (F) on bicuculline-induced tactile allodynia at 3 days after transfection. Values represent the allodynia score evaluated at each time point (\blacksquare) and withdrawal threshold (\diamond) (mean \pm S.E., N=7-12). * P <0.01 compared with the corresponding control values just prior to bicuculline injection, as determined by analysis of variance followed by a paired t -test. Area-under-the curve (AUC) is the area under the allodynia score and withdrawal threshold against time curve. $^\dagger P$ <0.01 compared with AUC 3 days after injection of HVJ-E and mutant siRNA, as determined by analysis of variance followed by Tukey's test.

Figure 5. Effect of transfection of siRNA for GlyR $\alpha 3$ on tactile allodynia induced by pCPT-cGMP. Tactile allodynia induced by pCPT-cGMP (0.29 nmol; 5 μ l of 58.0 μ M/mouse, i.t.) in mice transfected with HVJ-E (A), mutant siRNA (B) or GlyR $\alpha 3$

siRNA (C), 3 days after transfection, and 12 days after transfection of GlyR $\alpha 3$ siRNA (D). Values represent the allodynia score assessed by the paintbrush test evaluated at each time point (■) and the withdrawal threshold assessed by the von Frey hair test (◇) (mean \pm S.E., N=8-11). * P <0.01 compared with the corresponding control values just prior to pCPT-cGMP injection, as determined by analysis of variance followed by a paired t -test. † P <0.01 compared with AUC 3 days after injection of HVJ-E, mutant siRNA and 12 days after injection of GlyR $\alpha 3$ siRNA, as determined by analysis of variance followed by Tukey's test.

Figure 6. Effect of transfection of siRNA for GlyR $\alpha 3$ on tactile allodynia induced by PAF, glutamate and SIN-1. Tactile allodynia induced by PAF (0.19 fmol; 5 μ l of 38 pM/mouse, i.t., A, B), glutamate (5.0 pmol; 5 μ l of 1 μ M/mouse, i.t., C, D) or SIN-1 (48.4 nmol; 5 μ l of 9.7 mM/mouse, i.t., E, F) in mice transfected with HVJ-E (A, C, E), mutant siRNA (A, C, E) or GlyR $\alpha 3$ siRNA (B, D, F) 3 days after transfection. Values represent the allodynia score assessed by the paintbrush test evaluated at each time point (■) and the withdrawal threshold assessed by the von Frey hair test (◇) (mean \pm S.E., N=8). * P <0.01 compared with the corresponding control values just prior to the injection of PAF (A), glutamate (C) or SIN-1 (E), as determined by analysis of variance

followed by a paired *t*-test. † $P < 0.01$ compared with AUC at 3 days after injection of HVJ-E and mutant siRNA, as determined by analysis of variance followed by Tukey's test.

Table 1 Summary of information of various drugs used in this study.

Drugs and Abbreviations	Chemical name	Mechanisms of action	Route of administration	References
L-Arginine		Precursor of NO (substrate for NOS)	i.t.	[3, 6]
D-Arginine		Negative control for L-arginine (not a substrate for NOS)	i.t.	[6]
SIN-1	5-amino-3-morpholinyl-1,2,3-oxadiazolium chloride	NO donor	i.t.	[3, 50]
NOC-18	3,3- <i>bis</i> (aminoethyl)-1-hydroxy-2-oxo-1-triazene	NO donor	i.t.	[25, 26]
pCPT-cGMP	8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt	Membrane-permeable and phosphodiesterase stable analog of cGMP	i.t.	[48]
7-NINA	7-nitroindazole monosodium salt	Relatively selective inhibitor of nNOS <i>in vivo</i>	i.t.	[35]
TRIM	1-[2-(trifluoromethyl)phenyl]imidazole	Relatively selective nNOS inhibitor cf. eNOS <i>in vitro</i> and <i>in vivo</i>	i.t.	[35]
Hemoglobin		NO scavenger	i.t.	[5, 50]
Carboxy- PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt	NO scavenger	i.t.	[5]
ODQ	1 <i>H</i> [1,2,4]oxadiazolo[4,3- <i>a</i>]quinoxalin-1-one	Potent and selective inhibitor of NO-sensitive G-cyclase	i.t.	[3, 16, 50]
NS2028	4 <i>H</i> -8-bromo-1,2,4-oxadiazolo[3,4- <i>d</i>]benz[<i>b</i>][1,4]oxazin-1-one	Specific and irreversible G-cyclase inhibitor	i.t.	[40]

(Table 1 continued)

Drugs and Abbreviations	Chemical name	Mechanisms of action	Route of administration	References
Rp-8-pCPT-cGMPS	8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphorothionate, Rp-diastereomer sodium salt	Membrane-permeable, potent selective protein kinase G (PKG, G1 α , 1 β) inhibitor	i.t.	[52, 53]
KT5823	(9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1 <i>H</i> -diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester	Cell-permeable, selective, PKG inhibitor	i.t.	[48]

i.t. intrathecal injectio

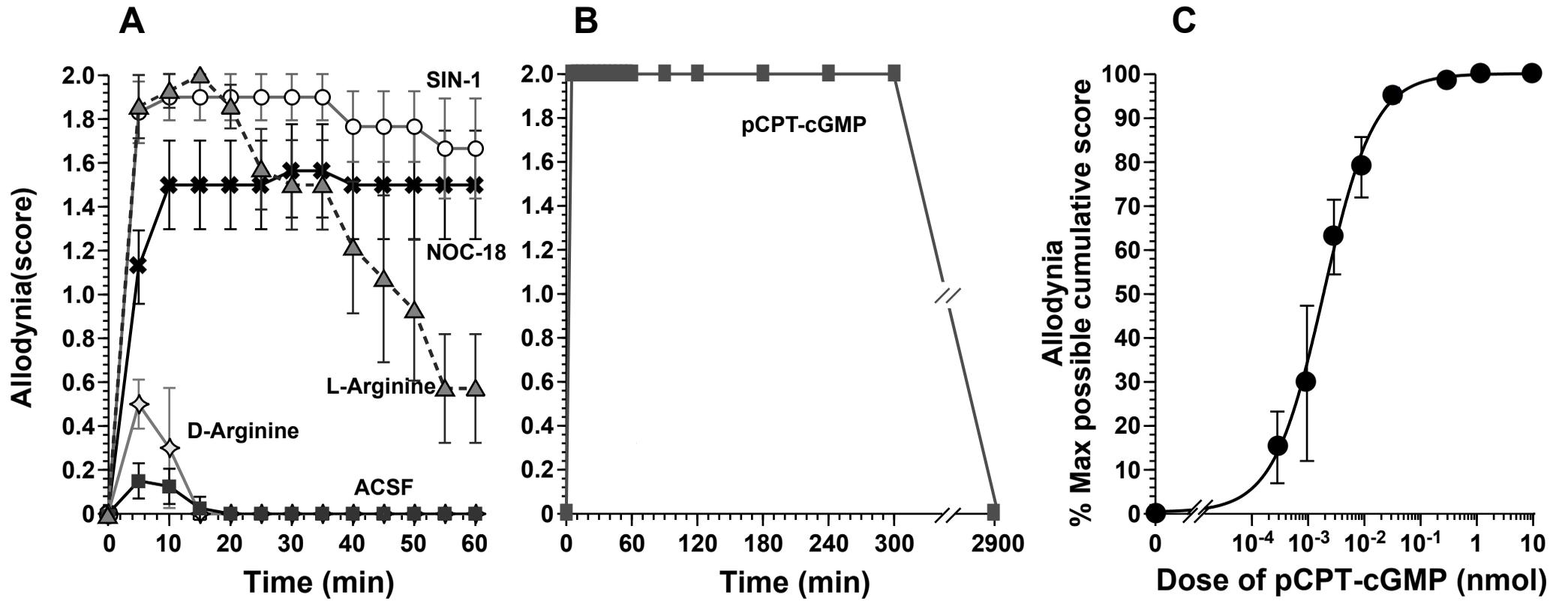


Fig. 1.

Pain

Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)

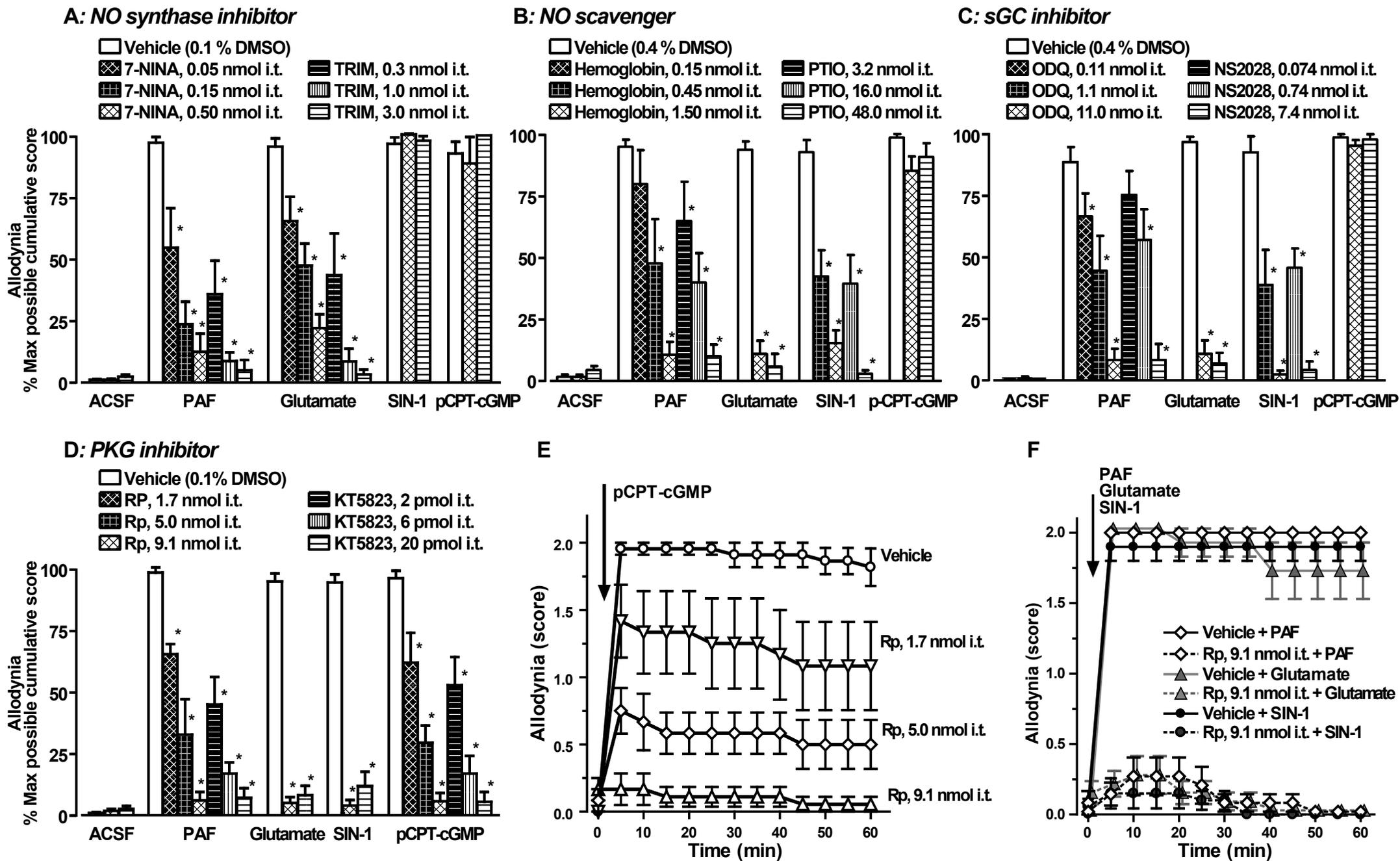


Fig. 2.

Pain
Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)

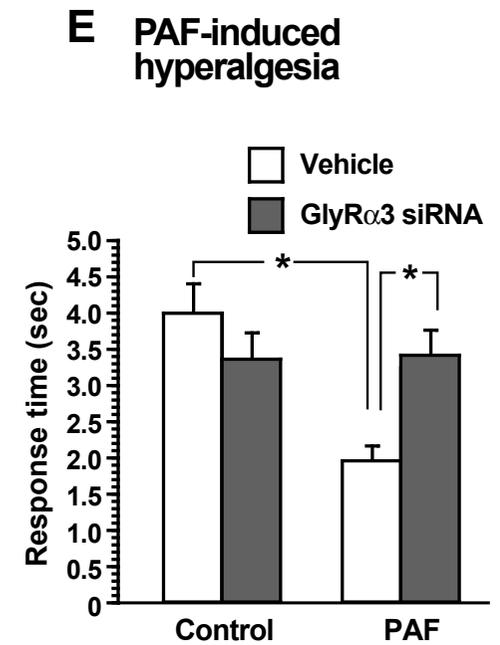
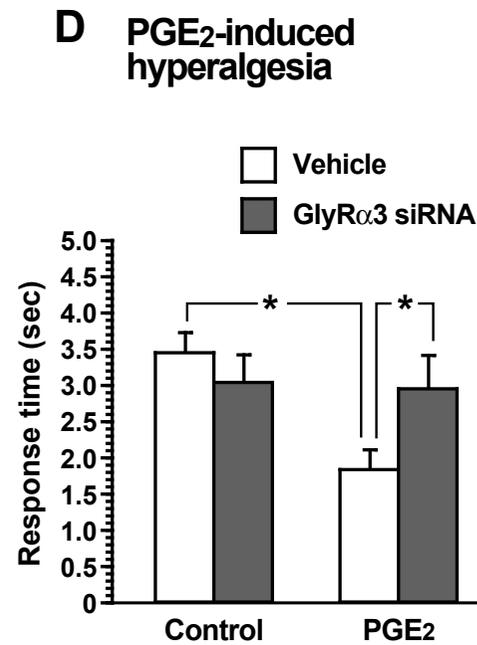
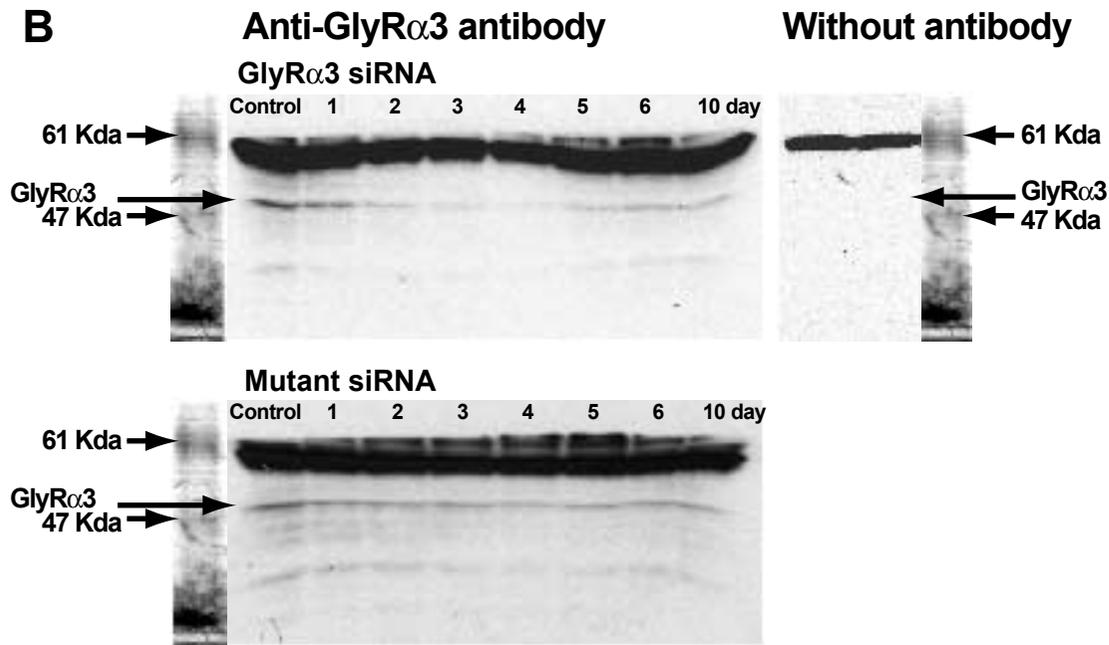
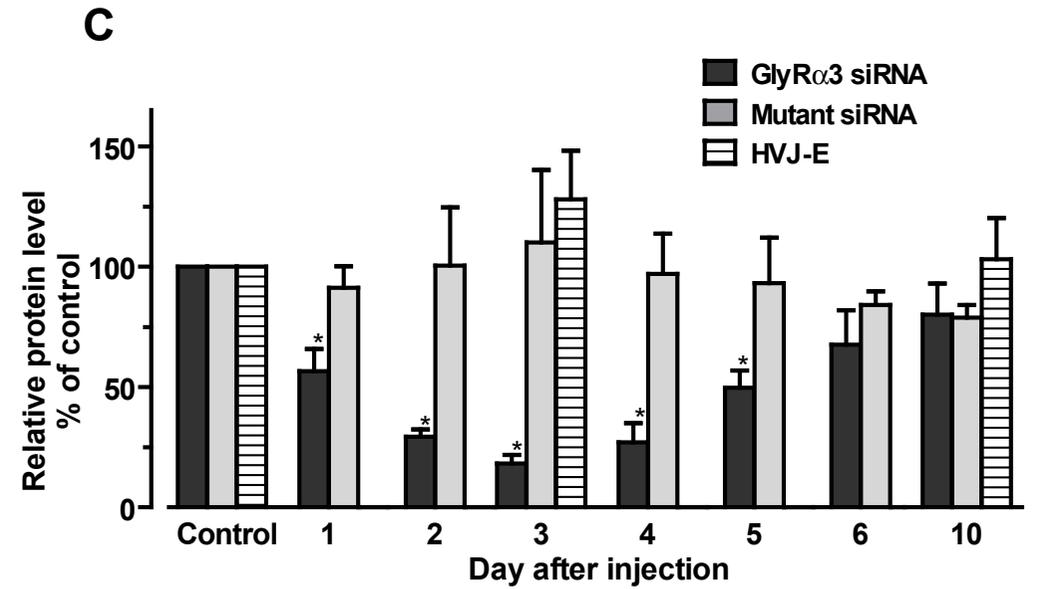
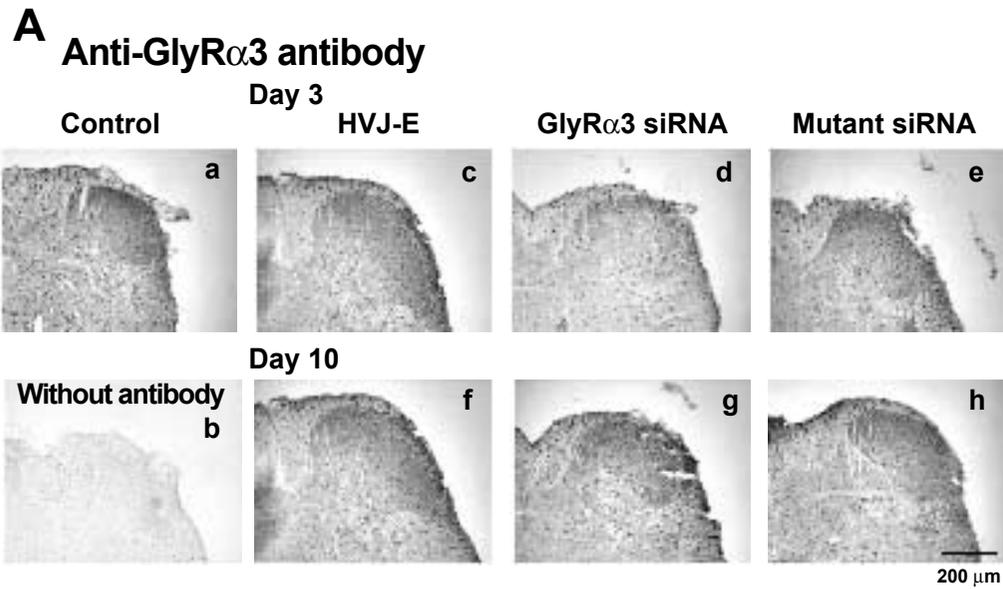


Fig. 3. Pain
Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)

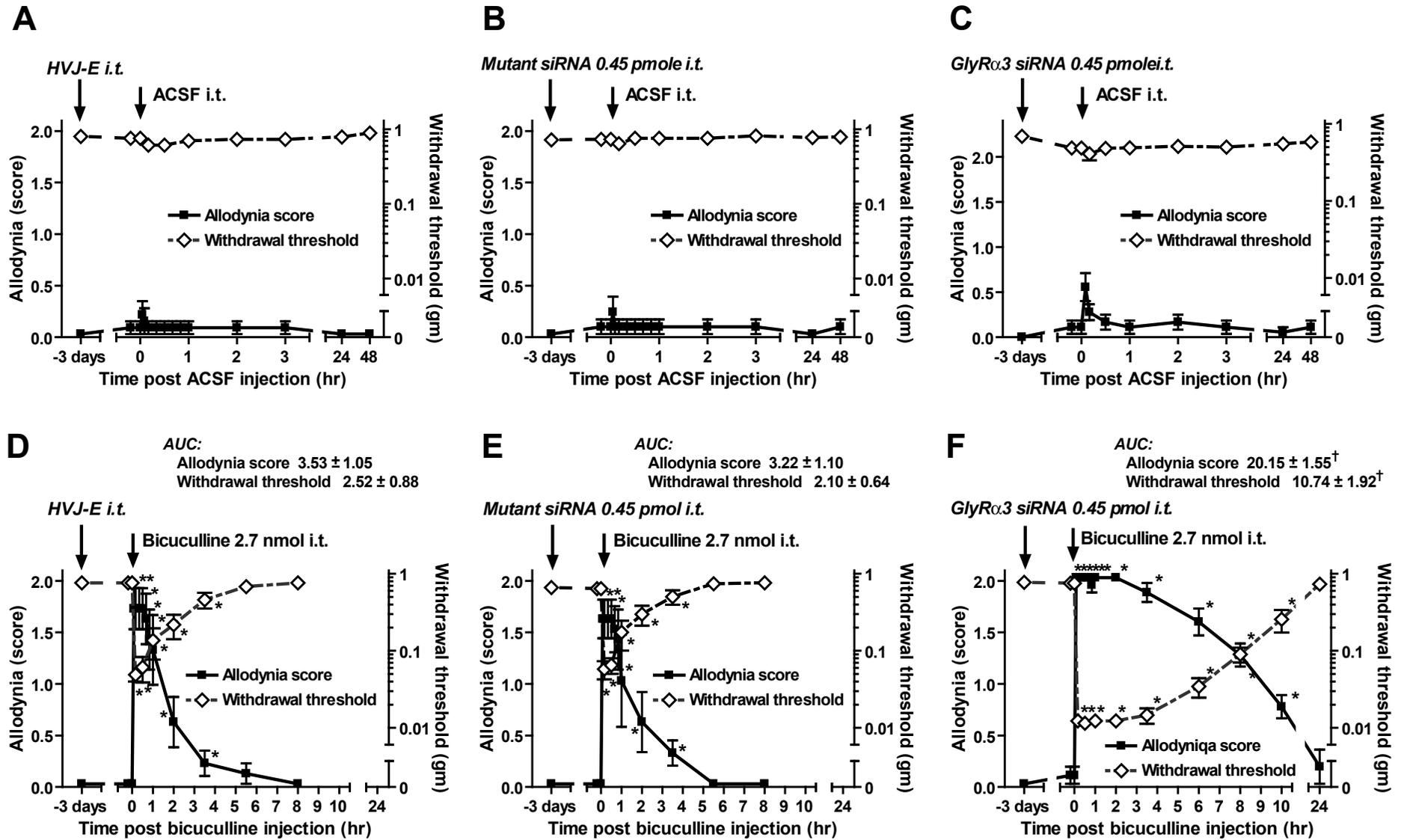


Fig. 4.

Pain

Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)

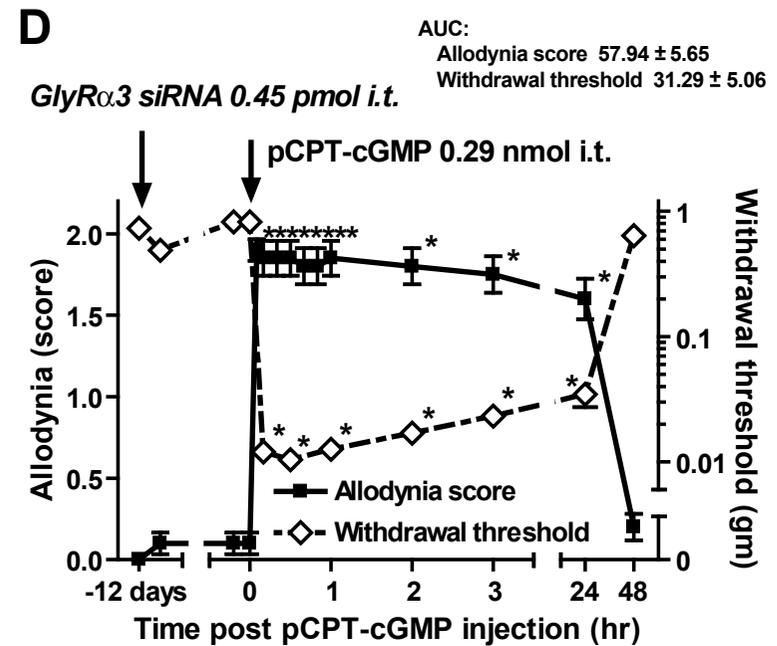
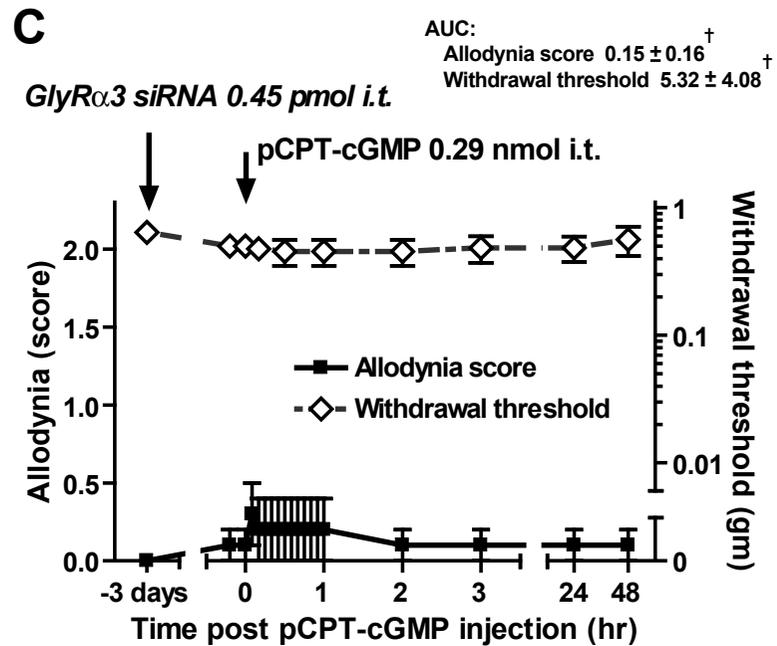
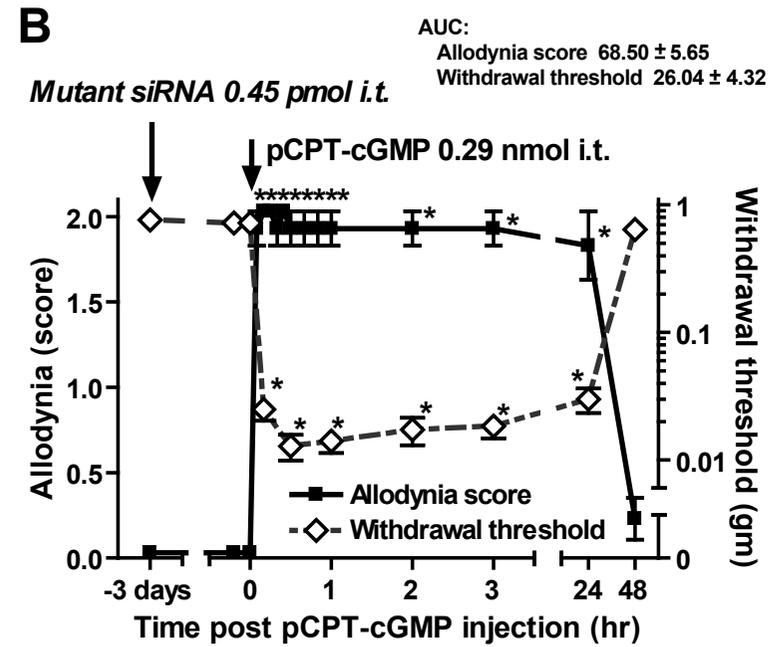
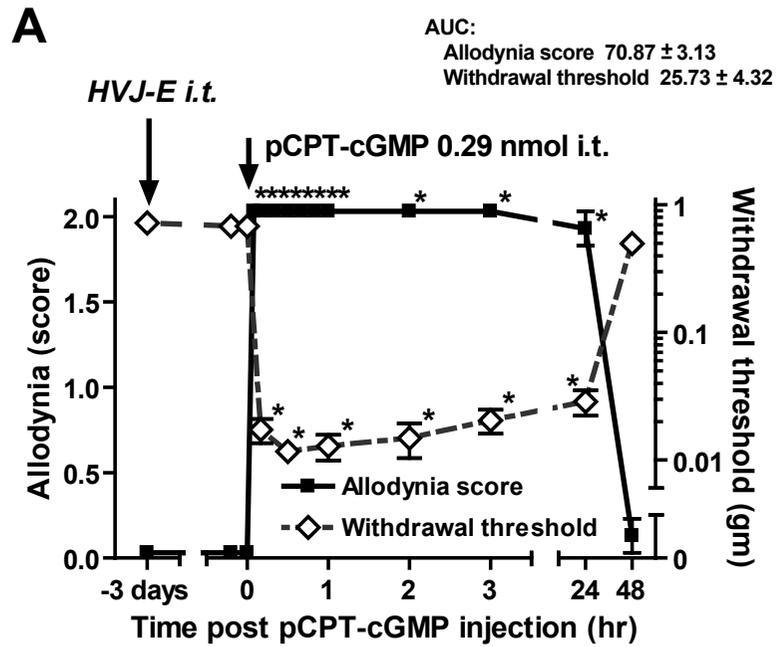


Fig. 5.
Pain
Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)

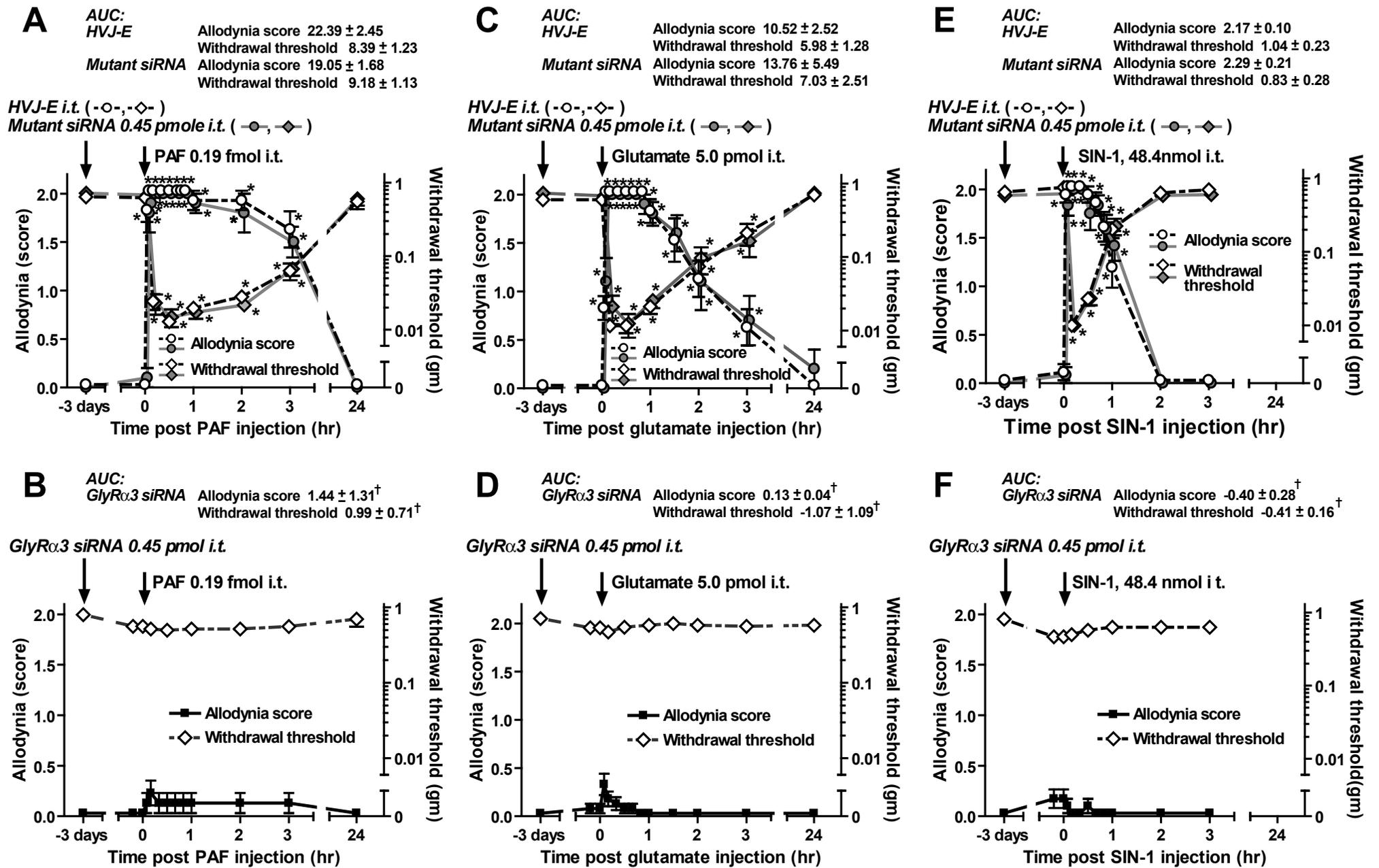


Fig. 6.

Pain

Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice (by K. Morita et al.)