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RESEARCH PAPER

Spinal neuronal NOS activation mediates sigma-1 receptor-induced mechanical and thermal hypersensitivity in mice: involvement of PKC-dependent GluN1 phosphorylation

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BACKGROUND AND PURPOSE

We recently demonstrated that activation of the spinal sigma-1 receptor induces mechanical and thermal hypersensitivity via calcium-dependent second messenger cascades and phosphorylation of the spinal NMDA receptor GluN1 subunit (pGluN1). Here we examined the role of NO in this process, as it plays a critical role in PKC-mediated calcium signalling and the potentiation of NMDA receptor function.

EXPERIMENTAL APPROACH

The effects of intrathecal (i.t.) pretreatment with nNOS inhibitors on PRE084 (sigma-1 receptor agonist)-induced pain were assessed in mice by use of mechanical allodynia and thermal hyperalgesia tests. Western blot analysis, immunoprecipitation and immunohistochemical techniques were used to determine effects of these treatments on spinal pGluN1-immunoreactive (ir) cells, whether PRE084 induces a time-dependent modification of nNOS activity in the dorsal horn, and if any changes in nNOS activity can be blocked by sigma-1 receptor, calcineurin or soluble guanylyl cyclase (sGC) inhibitors.

KEY RESULTS

PRE084, injected i.t., induced mechanical and thermal hypersensitivity, and increased the number of PKC- and PKA-dependent pGluN1-ir cells in spinal cord. This PRE084-induced hypersensitivity and increase in PKC-dependent pGluN1 expression were blocked by pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME) or 7-nitroindazole (7-NI). PRE084 also time-dependently decreased the ratio of phosphorylated nNOS (pnNOS) to nNOS expression and the number of spinal pnNOS-ir cells. This decrease in pnNOS was prevented by BD1047, a sigma-1 receptor antagonist and cyclosporin A, a calcineurin inhibitor, but not by a sGC inhibitor.

CONCLUSIONS AND IMPLICATIONS

Spinal sigma-1 receptor-induced sensitization is mediated by an increase in nNOS activity, which is associated with an NO-induced increase in PKC-dependent pGluN1 expression.

Abbreviations

7-NI, 7-nitroindazole; BD1047, *N*-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine dihydrobromide; CsA, cyclosporin A; DAG, diacyl glycerol; iNOS, inducible NOS; IP3, inositol triphosphate; L-NAME, N^G-L-nitro-arginine methyl ester; nNOS, neuronal NOS; NO, nitric oxide; ODQ, 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; pnNOS, phosphorylated form of nNOS; pGluN1, phosphorylated NMDA receptor GluN1 subunit; PWF, paw withdrawal frequency; sGC, soluble guanylyl cyclase

Introduction

The sigma-1 receptor has recently been identified as a unique ligand-regulated molecular chaperone in the endoplasmic reticulum of cells, and has been implicated in a myriad of cellular functions and biological processes (Collier *et al.*, 2007; Maurice and Su, 2009). Previous studies from our laboratories have demonstrated that the spinal sigma-1 receptor plays a pronociceptive role in formalin-induced pain (Kim *et al.*, 2006), and that the direct activation of spinal sigma-1 receptors using intrathecal (i.t.) injection of agonists enhances the response to peripheral mechanical stimuli, which is closely associated with calcium-dependent second messenger cascades, including PKC (Roh *et al.*, 2008a; 2010). In addition, we have shown that the activation of sigma-1 receptors increases PKC- and PKA-dependent phosphorylation of the NMDA receptor GluN1 (NR1) subunit (pGluN1) in the spinal cord dorsal horn, which results in the potentiation of i.t. NMDA injection-evoked spontaneous pain behaviour (Kim *et al.*, 2008). Moreover, several studies including a previous investigation from our laboratories have shown that the blockade of spinal sigma-1 receptors using i.t. injection of the sigma-1 receptor antagonist, BD1047 or using sigma-1 receptor knockout mice reduces the development of neuropathic pain and blocks the nerve injury-induced increase of phosphorylation of extracellular signal-regulated kinase (pERK) as well as pGluN1 in the spinal dorsal horn (Roh *et al.*, 2008b; de la Puente *et al.*, 2009). Despite this work, the precise mechanisms underlying sigma-1 receptor-mediated pain induction and its alteration of cellular activity including pGluN1 remains to be fully examined.

Nitric oxide (NO) is generated from L-arginine and molecular oxygen by the activity of three distinct NOS isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Palmer *et al.*, 1988). Importantly, NO plays a critical role in modulating synaptic transmission in both the central and peripheral nervous system (Meller and Gebhart, 1993). It has been well demonstrated that NO produced by nNOS in spinal cord participates in the early induction (Levy and Zochodne, 2004) and/or the maintenance of neuropathic pain (Xu *et al.*, 2007). In addition, the physical association of nNOS and the NMDA receptor with postsynaptic density protein-95 (PSD-95) through their specialized PDZ domains helps to explain the preferential link between NMDA receptors and NO production (Brenman *et al.*, 1996; Garthwaite, 2008). These findings raised the possibility that the action of spinal sigma-1 receptors on NMDA receptor function (pGLUN1 modulation) could be closely associated with nNOS activation in spinal cord leading to increased production of NO.

In terms of potential mechanisms by which sigma-1 receptors might potentiate NO, it is known that nNOS possesses several putative sites for phosphorylation in relation to the regulation by Ca^{2+}/calmodulin (Ca^{2+}/CAM). This phosphorylation is regulated by some kinases and phosphatases, for example, PKA, PKC, PKG, Ca^{2+}/CAM-dependent protein kinase (CaMK) II, phosphatase I, which can affect nNOS activity differently by phosphorylation at separate sites on the nNOS isoform or by differential effects on phosphorylation at the same site (Garthwaite, 2008; Zhou and Zhu, 2009). In this regard, CaMKII phosphorylates nNOS at Ser847, which reduces nNOS activity by inhibiting Ca^{2+}/CaM binding. In contrast, protein phosphatase 1 decreases the level of phosphorylation of nNOS (pnNOS) at Ser847, leading to an increase of nNOS activity (Rameau *et al.*, 2004; Zhou and Zhu, 2009). These results imply that the decrease in pnNOS possibly represents an increase in nNOS activity, which subsequently induces up-regulation of NO production. Although it was well documented that an increase in pnNOS at Ser847 results in a neuroprotective effect during cerebral ischaemia (Osuka *et al.*, 2002; Zhou *et al.*, 2008), the potential effect of changes in pnNOS on pain modulation in the spinal cord, particularly in relation to sigma-1 receptor-induced mechanical and thermal hypersensitivity, has not been examined.

In this regard, the present study was designed to investigate the potential relationship between sigma-1 receptor-induced mechanical and thermal hypersensitivity and NO signalling via nNOS activation in the spinal cord. Thus, we examined whether: (i) i.t. pretreatment with an nNOS inhibitor could reduce sigma-1 receptor-mediated pain induction by affecting the sigma-1 receptor-induced PKC- and PKA-dependent increase in spinal pGluN1-ir cells; (ii) the activation of sigma-1 receptors results in a time-dependent modification of nNOS activity (i.e. a decrease in pnNOS or a reduction of nNOS linked to PSD95) in the spinal cord dorsal horn; and (iii) this change in nNOS activity could be blocked by i.t. pretreatment with a sigma-1 receptor antagonist, a calcineurin (CN) inhibitor or a soluble guanylyl cyclase (sGC) inhibitor, respectively.

Methods

Experimental animals

Male ICR mice (20–25 g) were purchased from the Laboratory Animal Center of Seoul National University (Seoul, Republic of Korea). They had free access to food and water and were maintained in temperature and light controlled rooms (23 ± 2°C, 12/12 h light/dark cycle with lights on at 8:00 a.m.) for at least 1 week prior to beginning an experiment. All animal care and experimental procedures complied with the Guidelines of the NIH (1985) and were approved by the SNU Animal Care and Use Committee.

Drugs and i.t. administration

The following drugs were used: 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate (PRE084, 3 nmol), a sigma-1 receptor agonist; N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine dihydro-bromide (BD1047, 100 nmol), a sigma-1 receptor antagonist; N^G-nitro-L-arginine methyl ester (L-NAME, 3, 10 and 100 nmol), a non-specific NOS inhibitor; 7-nitroindazole (7-NI; 10, 100 and 200 nmol), a specific nNOS inhibitor; cyclosporin A (CsA; 20 nmol), a CN inhibitor; 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; 300 pmol), a sGC inhibitor. L-NAME, 7-NI, CsA and ODQ were supplied by Sigma-Aldrich (St. Louis, MO, USA). PRE084 and BD1047 were supplied by Tocris Cookson Ltd. (Bristol, UK). The doses of PRE084 and BD1047 used were based on our previous study showing that these doses produce maximal effects with no detectable side effects (Roh *et al.*, 2008a). The doses of L-NAME, 7-NI, CsA and ODQ were selected based on doses previously used in the literature including our previous study (Karlsson *et al.*, 2004; Chu *et al.*, 2005; Yoon *et al.*, 2008; Tanabe *et al.*, 2009). PRE084, BD1047, L-NAME, and ODQ were dissolved in physiological saline and all drugs were administered i.t.. 7-NI and CsA were dissolved in 5% DMSO, then, diluted in Corn oil. All drugs were administered 10 min before PRE084. Paw withdrawal responses to von Frey stimulation and the latency responses to heat stimuli (see below) were measured before and 30, 60 and 120 min after treatment with PRE084 (or saline) in all groups of mice.

Drugs were dissolved in 5 μL of vehicle. Intrathecal (i.t.) injections were made into the L5–L6 intervertebral space of unanaesthetized mice using a 10 μL Hamilton syringe connected to a 30-gauge needle as previously described by Hylden and Wilcox (1980). The flick of the tail was considered indicative of a successful i.t. injection. The control group received an i.t. injection of vehicle. Animals were randomly assigned to experimental groups and subsequent drug treatment and analysis were performed blindly.

Mechanical allodynia assay

Sensitization to innocuous mechanical stimulation (mechanical allodynia) was examined with von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) as described in our previous study (Roh *et al.*, 2008a). Based on this study, the 0.16 g von Frey filament was selected for testing. This von Frey filament was applied from underneath the metal mesh flooring to each hind paw. The filament was applied 10 times to each paw with each application separated by 10 s intervals. The number of paw withdrawal responses following each von Frey stimulus was then counted. The results of mechanical response testing in each experimental animal were expressed as a % of the paw withdrawal response frequency (PWF, %), which represented the percentage of paw withdrawals out of a maximum of 20 (right hind paw-10 and left hind paw-10).

Thermal hyperalgesia test

Sensitization to noxious heat stimulation (thermal hyperalgesia) was examined with a hot-plate apparatus (Model-35100, Ugo Basile, Comerio, Italy; Duman *et al.*, 2006; Milano *et al.*, 2008). The temperature of plate was maintained at 55 ± 0.5°C. Animals were placed into an acrylic cylinder (20 cm in diameter, 25 cm high) on the heated surface, and the time (in s) between placement and shaking or licking or lifting of their hind paws or jumping (whichever occurred first), was recorded as the latency responses (s). Baseline latency responses (8–12 s) were determined before experimental treatment. The latency responses were then measured 30, 60 and 120 min after treatment with PRE084 (or saline) in each experimental animal. The test was duplicated in each animal, and the mean latency response was calculated. Cut-off time in the absence of a response was set at 20 s to minimize tissue damage.

Paw pinch-induced spinal Fos expression

In another set of experiments, we quantified the number of Fos-ir cells in the spinal cord dorsal horn induced by a paw pinch stimulus and examined the effect of i.t. injection of drugs on this expression. Mice were anaesthetized with 5% isoflurane before application of the paw pinch to avoid any potential stress response or escape response. The right hind paw was pinched with a haemostatic forceps for 10 min (Lee *et al.*, 1992; Roh *et al.*, 2008a). Eight experimental groups were as follows: (i) anaesthesia only; (ii) PRE084 only (no pinch stimulation); (iii) paw pinch; (iv) PRE084 (3 nmol) + pinch; (v) L-NAME (100 nmol) + PRE084 + pinch; (vi) L-NAME (100 nmol) + pinch; (vii) 7-NI (100 nmol) + PRE 084+ pinch; and (viii) 7-NI (100 nmol) + pinch. The PRE084 was administered 30 min before pinch stimulation (Roh *et al.*, 2008a), and L-NAME and 7-NI were administered 10 min before i.t. PRE084 injection.

Two hours after paw-pinch stimulation, the mice were humanely killed and perfused with fixative, and Fos immunohistochemical staining was subsequently performed on spinal cord sections according to the method detailed in our previous reports (Kwon *et al.*, 2001; Roh *et al.*, 2008a). After postfixation and cryoprotection, a series of frozen sections (40 μm thickness) were cut through the L_{4-6} segments of the spinal cord and processed for Fos immunohistochemistry as previously described (Roh *et al.*, 2008a).

Western blot analysis of pnNOS and nNOS

Mice were deeply anaesthetized with 5% isoflurane at one time point before and at several time points (30, 60 and 120 min) after i.t. injection of PRE084 (3 nmol) to determine the time-dependent effect of sigma-1 receptor activation on the change in pnNOS to nNOS expression using immunoblot analysis (n = 5 at each time point group, total n = 20). The spinal cord was

extracted by pressure expulsion with air into an ice-cooled, saline-filled glass dish and snap-frozen in liquid nitrogen. In order to verify the location of the $L_{4–6}$ spinal cord segments for Western blotting, we identified the attachment site of each spinal nerve in anaesthetized mice. In addition, spinal segments were separated into left and right halves under a neurosurgical microscope. The spinal cord was subsequently further subdivided into dorsal and ventral halves by cutting straight across from the central canal laterally to a midpoint in the white matter. The right and left spinal cord dorsal horns were subsequently used for Western blot analysis. This method allowed us to analyse the changes in sigma-1 receptor agonist-induced nNOS and pnNOS selectively in the spinal cord dorsal horn. The L_{4-6} spinal cord dorsal segments were homogenized in buffer containing 1 M Tris (pH 7.5), 1% NP-40, 0.5 M EDTA (pH 7.5), 50 mM EGTA, 1 M dithiothreitol, 1 M benzanidine and 0.1 M PMSF. The total amount of protein in each sample was determined using the Bradford dye assay prior to loading on polyacrylamide gels. Spinal cord homogenates (20 μg protein) were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After the blots had been washed with TBST (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20), the membranes were blocked with 5% skimmed milk for 1 h and incubated at 4°C overnight with a primary antibody specific for β-actin (1:1000, loading control, Sigma), nNOS (1:1000, cat# 610311, BD Biosciences, San Jose, CA, USA) or for pnNOS (1:1000, cat# ab16650, Abcam Inc., Cambridge, MA, USA; this antibody is specific for mouse nNOS phosphorylated on serine 847). The membranes were washed and primary antibodies were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The positive pixel area of specific bands was measured with a computer-assisted image analysis system and normalized against the corresponding β-actin loading control bands. Then the ratio of pnNOS (Ser847) to nNOS expression was calculated. The mean value of the ratio of pnNOS to nNOS expression in animals prior to PRE084 injection (0 min) was set at 100%. Thus, the % change in pnNOS to nNOS expression in each time-point group was examined.

Co-immunoprecipitation for nNOS and PSD95

The interaction of nNOS with PSD95 in the spinal dorsal horn was analysed by immunoprecipitation and Western blotting ($n = 3$ in each group). Tissue homogenates were lysed with lysis buffer [1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) that contained 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, 2.5 mM Na4PO7, 100 mM NaF, 200 nM microcystin-lysine-arginine, and protease inhibitors] and the tissue lysates (300 μg) were mixed with 10 μg of rabbit anti-nNOS antibody (BD Biosciences). The samples were incubated for 4 h, mixed with Protein A/G PLUS-agarose immunoprecipitation reagent (Pierce, Rockford, IL, USA), and then incubated for an additional 12 h. The beads were washed four times, and the bound proteins were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analysed by Western blotting with mouse anti-PSD95 monoclonal antibody (1:1000, cat# P246, Sigma, St. Louis, MO, USA).

pnNOS immunohistochemistry

In a separate set of experiments, mice were anaesthetized with 5% isoflurane at one time point before and at several time points (30, 60 and 120 min) after i.t. injection of PRE084 (3 nmol) and perfused transcardially with calcium-free Tyrode's solution followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9). The spinal cords were removed immediately after perfusion, post-fixed in the identical fixative for 12 h and then placed in 30% sucrose in PBS (pH 7.4) overnight at 4°C. Serial transverse sections (40 μm) of the spinal cord were cut using a cryostat (Microm, Germany). Spinal L4–L6 tissue sections were processed for pnNOS (rabbit polyclonal anti-pnNOS-Ser847 antibody, 1:3000; Abcam Inc.) immunohistochemistry using the avidin-biotin-peroxidase complex (ABC) procedure as previously described (Osuka *et al.*, 2007). Visualization of the ABC complex was performed using 3,3-diaminobenzidine (Sigma) and the 3,3-diaminobenzidine reaction was intensified with 0.2% nickel chloride.

pGluN1 immunohistochemistry

The immunohistochemical procedures for pGluN1 antibody localization have been previously described in detail (Kim *et al.*, 2006; 2008; Roh *et al.*, 2008b). Thirty minutes after i.t. injection of PRE084 or vehicle, mice were deeply anaesthetized with 5% isoflurane and spinal cords were removed and processed using the same procedure described for pnNOS immunohistochemistry. Spinal L4–L6 tissue sections were processed immunohistochemically for the localization of PKC-dependent pGluN1 (Ser896, 1:1000, cat# 06–640, Upstate Biotechnology, Lake Placid, NY, USA; this antibody is specific for GluN1 phosphorylated on serine 896) or for PKA-dependent pGluN1 (Ser897, 1:1000, cat# 06–641, Upstate Biotechnology; this antibody is specific for GluN1 phosphorylated on serine 897) using the avidin-biotin-peroxidase complex (ABC) procedure as previously described.

Image analysis

Tissue sections were examined under a brightfield microscope (Zeiss Axioscope, Hallbergmoos, Germany) and five spinal cord sections from the L4-6 lumbar spinal cord segments were randomly selected from each animal, and subsequently scanned. Individual sections were digitized with 4096 grey levels using a cooled CCD camera (Micromax Kodak 1317; Princeton Instruments, Tucson, AZ, USA) connected to a computer-assisted image analysis system (Metamorph version 6.3r2; Molecular Devices Corporation, Downingtown, PA, USA). To maintain a constant threshold for each image and to compensate for subtle variability of the immunostaining, we only counted neurones that were at least 70% darker than the average grey level of each image after background subtraction and shading correction. The average number of Fos-ir, pGluN1-ir and pnNOS-ir cells per section from each animal was obtained and these values were averaged across each group and presented as group data. The expression of Fos, pGluN1 and pnNOS was quantified in the following three dorsal horn regions: (i) the superficial dorsal horn (SDH; laminae I and II); (ii) the nucleus proprius (NP; laminae III and IV); and (iii) the neck region (NECK, laminae V and VI).

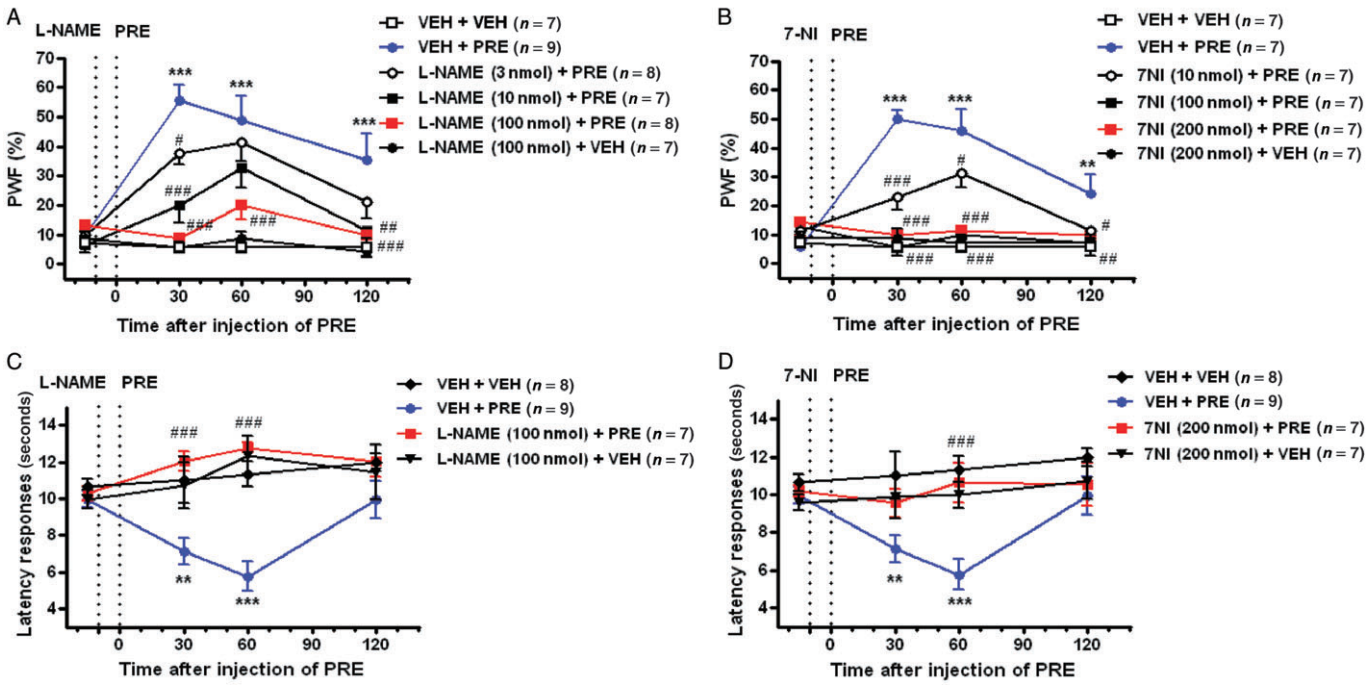


Figure 1

The effect of intrathecal (i.t.) administration of the NOS inhibitors, N^G-nitro-L- arginine methyl ester (L-NAME; a non-selective NOS inhibitor) or 7-nitroindazole (7-NI; a selective nNOS inhibitor) on the PRE084 (PRE; 3 nmol)-induced changes in the paw withdrawal frequency over time (PWF; %, A, B), and in the latency responses (seconds, C, D). Either L-NAME (A, C) or 7-NI (B, D) was applied 10 min before PRE084 injection, respectively. The PWF and the latency responses were examined at 30, 60, 120 min after PRE084 injection using a von-Frey filament (0.16 g), and hot plate test (55 ± 0.5°C), respectively. Repeated measures two-way ANOVA followed by Bonferroni's multiple comparison tests were performed (**$P < 0.01$ and ***$P < 0.001$ as compared with those of the VEH + VEH group and #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ as compared with those in the VEH + PRE group).

All analytical procedures described earlier were performed without the knowledge of the experimental conditions.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was performed using Prism 5.0 (Graph Pad Software, San Diego, CA, USA). Repeated measures two-way ANOVA was performed to determine overall differences in the time-course of all nociceptive behavioural tests. *Post hoc* analysis was performed using the Bonferroni's multiple comparison test in order to determine the *P*-value among experimental groups. One-way ANOVA followed by the Bonferroni's comparison test was used to determine differences in the number of spinal pGluN1-ir and pnNOS-ir cells (immunohistochemistry) and in the ratio of pnNOS to nNOS expression (Western blot assay) between experimental groups. A $P < 0.05$ was considered statistically significant.

Results

Effect of i.t. pretreatment with NOS inhibitors on PRE084-induced mechanical allodynia

The i.t. administration of PRE084 (3 nmol, VEH + PRE) significantly increased PWF to innocuous mechanical stimuli (mechanical allodynia) for the entire 120 min post-injection testing period as compared with those of VEH + VEH-treated group (Figure 1A and B). I.t. pretreatment with L-NAME (3, 10 and 100 nmol, Figure 1A), a non-selective NOS inhibitor or with 7-NI (10, 100 and 200 nmol, Figure 1B), a selective nNOS inhibitor dose-dependently suppressed this PRE084-induced increase in PWF. The i.t. injection of these inhibitors alone (L-NAME + VEH or 7-NI + VEH), in the absence of PRE084, did not affect PWF in comparison with the VEH + VEH group (Figure 1A and B).

Effect of i.t. pretreatment with NOS inhibitors on PRE084-induced thermal hyperalgesia

The i.t. injection of PRE084 (3 nmol, VEH + PRE) also decreased latency responses to noxious heat stimuli (thermal hyperalgesia) for the entire 60 min post-injection testing period as compared with that of the VEH + VEH treated group (Figure 1C and D). Pretreatment with L-NAME (100 nmol, i.t., Figure 1C) or with 7-NI (200 nmol, i.t., Figure 1D) blocked the PRE084-induced decrease in latency responses. The injection of these inhibitors alone (L-NAME + VEH or 7-NI + VEH) did not evoke a significant change in latency responses in comparison with the VEH + VEH group (Figure 1C and D).

Effect of i.t. pretreatment with NOS inhibitors on the PRE084-induced enhancement of Fos expression caused by a paw pinch stimulus

The normal control animals that merely received isoflurane treatment showed only a few Fos-ir neurones that were scattered in the SDH, NP and NECK regions of the dorsal horn of the spinal cord (Figure 2A, B). A diagram depicting the location of each of these three regions in the spinal dorsal horn is superimposed on the photomicrograph of the spinal cord illustrated in the first plate of Figure 2B. Right paw-pinch stimulation for 10 min significantly increased the number of Fos-ir cells in all regions of the ipsilateral spinal cord dorsal horn (Figure 2A, B), but had no effect on Fos expression in the contralateral dorsal horn (data not shown). PRE084 (3 nmol, i.t.) significantly enhanced paw pinch-induced Fos expression in the dorsal horn compared with that observed in the paw pinch only group (Figure 2A and B). Pretreatment with L-NAME (100 nmol, i.t.) or 7-NI (100 nmol, i.t.) completely blocked this PRE084-induced facilitatory effect on spinal Fos expression (Figure 2A, B). In contrast, L-NAME or 7-NI alone in the absence of PRE084 did not modify paw pinch-evoked Fos expression in the dorsal horn. Similarly, i.t. injection of PRE084 without paw pinch stimulation had no effect on the number of Fos-ir neurones in the dorsal horn compared with those present in normal animals (Figure 2A, B).

Effect of i.t. pretreatment with NOS inhibitors on the PRE084-induced increase in the number of pGluN1-ir cells in the spinal dorsal horn

The i.t. administration of PRE084 (3 nmol) significantly increased the number of PKC-dependent (Ser896) pGluN1-ir cells in the NP (laminae III-IV) and NECK regions (laminae V-VI) and PKA-dependent (Ser897) pGluN1-ir cells in the SDH (laminae I-II), NP and NECK regions of the spinal cord dorsal horn at the 30 min post-injection time point (Figures 3A, B and 4A, D). Pretreatment with L-NAME (100 nmol, i.t.) or 7-NI (100 nmol, i.t.) potently suppressed this PRE084-induced increase in the number of PKC-dependent (Ser896) pGluN1-ir neurones in the spinal cord dorsal horn, particularly in the NP and NECK regions (Figures 3A and 4B, C). By contrast, the PRE084-induced increased number of PKA-dependent (Ser897) pGluN1-ir neurones was not reduced by either L-NAME (100 nmol) or 7-NI (100 nmol) injection (Figures 3B and 4E, F). The i.t. injection of these inhibitors alone, in the absence of PRE084, did not affect spinal pGluN1 expression in comparison with the VEH + VEH group (Figure 3A and B).

Effect of i.t. PRE084 injection on the phosphorylation (Ser847) of nNOS and the binding of PSD-95 to nNOS in the spinal cord

PRE084 (i.t.) significantly decreased the ratio of pnNOS (Ser847) to nNOS expression at both the 30 and 60 min time points (Figure 5A). In addition, i.t. injection of PRE084 also reduced the amount of nNOS and PSD95 binding forms in the spinal cord dorsal horn at 30 and 60 min post-injection (Figure 5B). Moreover, the number of pnNOS-ir cells in the SDH (laminae I-II) region was dramatically decreased after i.t. PRE084 (3 nmol), especially 30 and 60 min post-injection (Figure 6A). Image analysis confirmed that a significant decrease in the number of pnNOS (Ser847)-ir neurones occurred 30 and 60 min after PRE084 injection as compared with that of the non-treatment group (0 min) (Figure 6B).

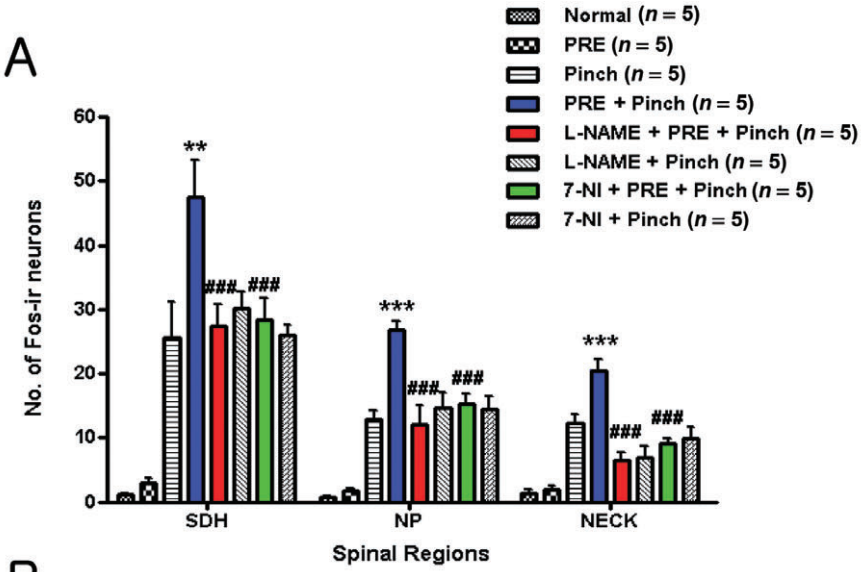
Effect of pretreatment with a sigma-1 receptor antagonist or a CN inhibitor on PRE084-induced mechanical and thermal hypersensitivity and decrease in pnNOS expression

Pretreatment with sigma-1 receptor antagonist, BD1047 (100 nmol, i.t.), completely abolished PRE084 (3 nmol)-induced mechanical and thermal hypersensitivity at both the 30 and 60 min time points (Figure 7A and B). Pretreatment with BD1047 alone (BD + VEH) did not alter the PWF and the latency responses. In addition, the PRE084-induced decrease in the number of pnNOS-ir neurones in the SDH (laminae I–II) region was prevented by pretreatment with BD1047 (Figure 7C).

Pretreatment with CN inhibitor, CsA (20 nmol, i.t.), significantly attenuated PRE084-induced mechanical and thermal hypersensitivity at all time points (Figure 8A and B). Pretreatment with CsA alone did not alter the PWF and the latency responses. The PRE084-induced decrease in the number of pnNOS-ir neurones in the SDH region was blocked by pretreatment with CsA (Figure 8C). Pretreatment with either BD1047 or CsA alone did not affect the number of pnNOS-ir cells in the spinal cord (Figures 7C and 8C).

Figure 2

The effect of intrathecal (i.t.) administration of the NOS inhibitors, L-NAME (a non-selective NOS inhibitor) or 7-nitroindazole (7-NI; a selective nNOS inhibitor) on PRE084 (PRE, 3 nmol)-induced enhancement of paw pinch-evoked spinal cord Fos expression. The number of Fos-immunoreactive (ir) neurones in the superficial dorsal horn (SDH; lamina I–II), in the nucleus proprius (NP, lamina III–IV) and in the neck region (NECK, lamina V–VI) of the spinal cord dorsal horn are depicted graphically in (A). A one-way ANOVA followed by Bonferroni's multiple comparison tests was performed (**$P < 0.01$ and ***$P < 0.001$ as compared with that of the Pinch group, and ###$P < 0.001$ as compared with that in the PRE + Pinch group). Photomicrographs of representative spinal cord sections are illustrated in (B). The white dotted lines in the first panel depict the location of each of the three regions analysed in the spinal cord dorsal horn. Normal control group (Normal), i.t. PRE084 alone-treatment group (PRE, 3nmol), paw pinch-stimulated group (Pinch), i.t. PRE084 treatment + paw pinch group (PRE + Pinch), i.t. L-NAME (100 nmol) + PRE084 treatment + paw pinch group (L-NAME + PRE + Pinch) and i.t. 7-NI (100 nmol) + PRE084 treatment + paw pinch group (7-NI + PRE + Pinch). The PRE084 was administered 30 min before pinch stimulation, and L-NAME and 7-NI were administered 10 min before PRE084 injection. Arrows indicate representative Fos-ir cells. Scale bar = 200 μm.


A
Normal (n = 5)
PRE (n = 5)
Pinch (n = 5)
PRE + Pinch (n = 5)
L-NAME + PRE + Pinch (n = 5)
L-NAME + Pinch (n = 5)
7-NI + PRE + Pinch (n = 5)
7-NI + Pinch (n = 5)
No. of Fos-ir neurons
60
50
40
30
20
10
0
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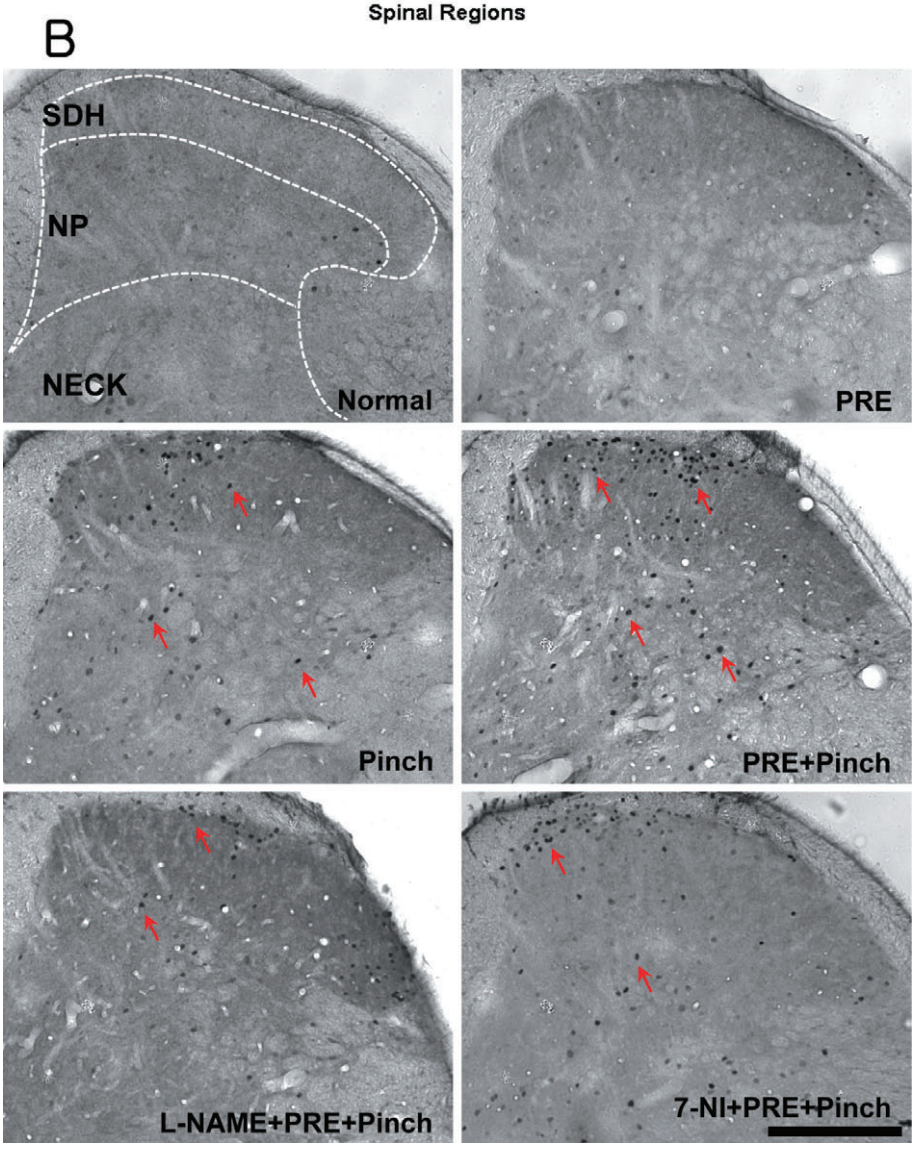
SDH
NP
NECK
Spinal Regions


B
SDH
NP
NECK
Normal
PRE
Pinch
PRE+Pinch
L-NAME+PRE+Pinch
7-NI+PRE+Pinch

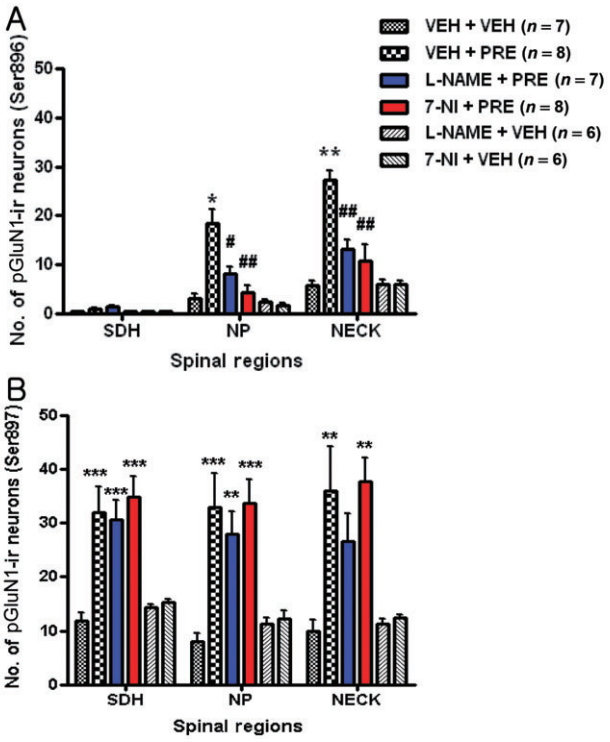


Figure 3

The effect of intrathecal (i.t.) administration of L-NAME (a non-selective NOS inhibitor, 100 nmol) or 7-nitroindazole (7-NI: a selective nNOS inhibitor, 100 nmol) on the i.t. PRE084 (PRE, 3 nmol)-induced increase in the number of PKC (A, Ser896)- and PKA (B, Ser897)-dependent GluN1 subunit phosphorylation (pGluN1)-immunoreactive (ir) cells in the spinal cord dorsal horn. A one-way ANOVA followed by Bonferroni's multiple comparison tests was performed on the data (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with those of VEH + VEH group and #$P < 0.05$ and ##$P < 0.01$ as compared with those of VEH + PRE group). SDH, superficial dorsal horn; NP, nucleus proprius; NECK, neck of dorsal horn.

Effect of i.t. pretreatment with a sGC inhibitor on PRE084-induced mechanical and thermal hypersensitivity and decrease in pnNOS expression

Pretreatment with the sGC inhibitor, ODQ (300 pmol, i.t.) significantly decreased PRE084-induced mechanical and thermal hypersensitivity (Figure 9A and B). Pretreatment with ODQ alone did not alter either PWF or latency responses or pnNOS expression. In contrast, the PRE084-induced decrease in the number of pnNOS-ir neurones was notably not modified by pretreatment with ODQ (Figure 9C).

Discussion and conclusions

Recent studies from our laboratories have demonstrated that i.t. administration of a sigma-1 receptor agonist (PRE084) significantly induced mechanical and thermal hypersensitivity to peripheral stimuli (Roh *et al.*, 2008b; 2010). These PRE084-mediated effects were associated with an increase in intracellular Ca^{2+} concentration through a PLC-IP3-PKC signalling pathway, which is also known to be closely linked to NO signalling via nNOS activation. The present study is the first to demonstrate that i.t. pretreatment with either the non-selective NOS inhibitor, L-NAME or the selective nNOS inhibitor, 7-NI reduced PRE084-mediated mechanical allodynic and thermal hyperalgesic behaviours as well as the PRE084-induced increase in paw pinch-evoked spinal cord Fos expression (i.e. this increased Fos expression reflects the PRE084-induced mechanical hyperalgesia). These results indicate that NO signalling via nNOS activation also serves as an intrinsic mechanism leading to the initiation of the central sensitization related to sigma-1 receptor activity.

It is well recognized that NO is synthesized by nNOS in the nervous system, which links to NMDA receptors through a postsynaptic density protein, PSD-95 (Xu *et al.*, 2007). Extracellular Ca^{2+} influx into neuronal cells via NMDA receptor activation increases nNOS activity effectively through binding of Ca^{2+}/calmodulin to the nNOS, and dephosphorylation occurs by Ca^{2+}/calmodulin-dependent nNOS phosphatase, a CN (Zhou and Zhu, 2009). In this regard, we determined that i.t. PRE084 modified the ratio of pnNOS (phosphorylated form of nNOS) to nNOS expression and decreased the number of pnNOS-ir cells in the spinal cord dorsal horn. Moreover, the sigma-1 receptor-induced reduction in the PSD-95 binding form of nNOS was confirmed using a co-immunoprecipitation method. These results demonstrate that activation of the NO signalling pathway stimulated by i.t. PRE084 is closely linked to decreases in the phosphorylated form of nNOS and its separation from the PSD-95 protein. That is, PRE084-induced sigma-1 receptor activation leads to a time-dependent stimulant effect on NO signalling, which ultimately induces mechanical allodynia and thermal hyperalgesia.

In the present study it was also found that the activation of nNOS in the spinal cord contributes to the increase in PKC-dependent, but not PKA-dependent, pGluN1 expression in the spinal cord dorsal horn. NO has been reported to activate PKC in the heart (Ping *et al.*, 1999). Following an ischaemic stimulus (endogenous increase of NO) or after treatment with NO-releasing agents (exogenous increase of NO), NO caused an isoform-selective activation of PKCε in rabbit (Ping *et al.*, 1999). Our recent study also demonstrated that the PRE084-induced increase in spinal pGluN1 expression is mediated by the PKCα and PKCε isoforms, but not the PKCζ isoform. The PKCα and PKCε isoforms in turn contribute to mechanical allodynia (Roh *et al.*, 2010). PKCα is a classical subtype which is both calcium and diacylglycerol (DAG)-dependent, while PKCε is an atypical subtype that is calcium-independent and DAG-dependent (Yonezawa *et al.*, 2009). Because NO signalling could be associated with DAG as well as calcium, the activation of PKCα and PKCε by NO signalling could have a strong influence on the sigma-1 receptor-induced increase in the PKC-dependent pGluN1 expression.

The precise mechanism by which an increase in NO induces a PKC-dependent modification of pGluN1 is a complex and requires further extensive investigation. NO signalling is carried out by at least two separate pathways. First, NO stimulates sGC to increase cGMP, which in turn modulates a variety of downstream signalling targets

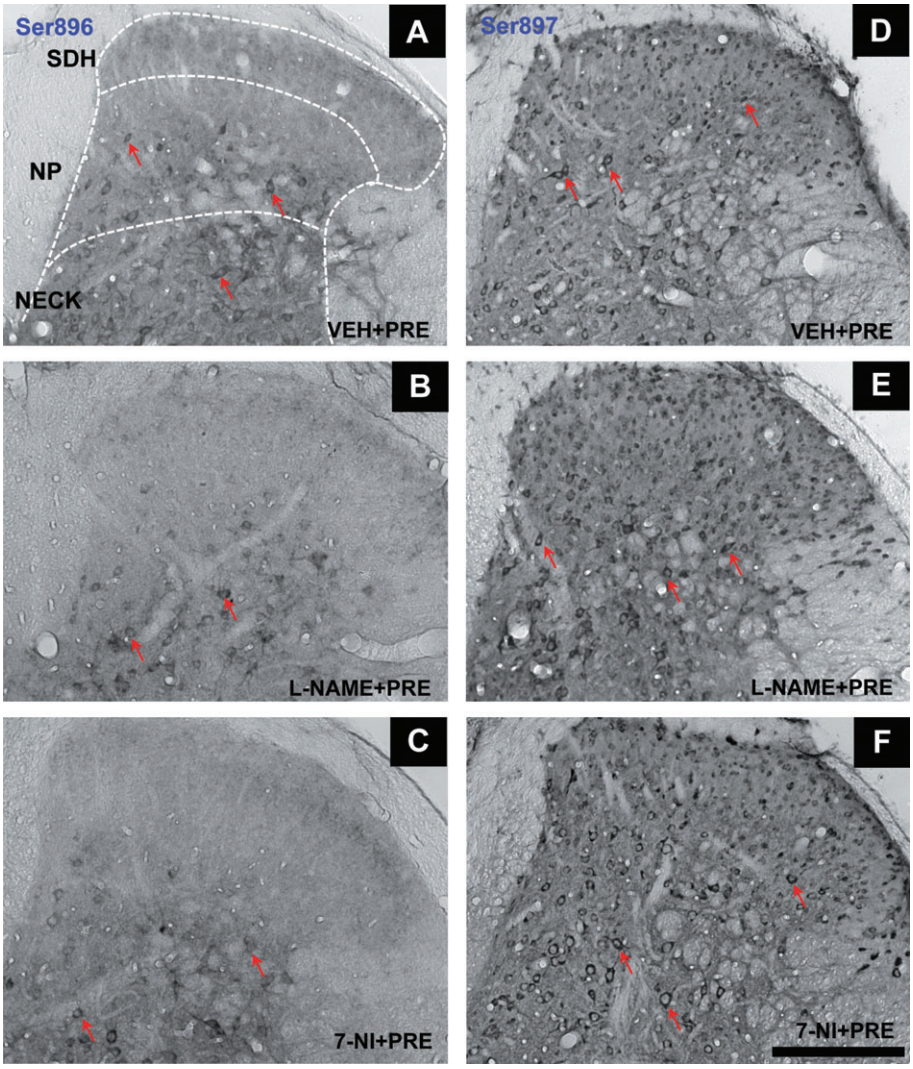


Figure 4

Photomicrographs of representative L4–L5 spinal cord sections illustrating PKC-dependent (Ser896) and PKA-dependent (Ser897) pGluN1-immunoreactive (ir) cells in the spinal cord dorsal horn induced by intrathecal (i.t.) administration of PRE084 (PRE, 3 nmol; A, D) alone, or following pretreatment with L-NAME (100 nmol; B, E) or 7-nitroindazole (7-NI; 100 nmol, C, F). Red arrows indicate representative pGluN1-ir cells. Scale bar = 200 μm. SDH, superficial dorsal horn; NP, nucleus proprius; NECK, neck of dorsal horn. The white dotted lines in (A) depict the location of each of these three regions in the spinal cord dorsal horn.

(Koesling *et al.*, 2004). Second, NO can directly modulate the functions of many proteins by reacting with haeme groups, sulphur-iron clusters and thiol moieties (Hess *et al.*, 2005; Miyamoto *et al.*, 2009). Thus, NO can affect PKC activity directly or indirectly by binding to proteins involved in PKC modulation. Furthermore, cGMP is known to be a particularly important second messenger for NO-dependent signalling (Schmidtko *et al.*, 2008; 2009). We suggest that the sigma-1 receptor-induced increase in NO signalling is mediated by a cGMP signal pathway, but not by a direct action of NO on PKC activity, as the sGC inhibitor, ODQ, totally attenuated the PRE084-induced mechanical and thermal hypersensitivity in this study. Moreover, while Ca^{2+} signalling is also essential for activation of nNOS and catalytic activation of Ca^{2+}-dependent PKC, the activity of PKA is dependent on the concentration of cAMP, but not the concentration of Ca^{2+} within the cell. Therefore, the sigma-1 receptor-induced increase in intracellular Ca^{2+} concentration activates a NO-sGC signalling pathway originating from nNOS, which in turn affects PKC-dependent, but not PKA-dependent, pGluN1 expression in the spinal cord dorsal horn (see Figure 10 for a schematic summary of this putative mechanism).

The immunohistochemical data also show that the PRE084-induced expression pattern of pnNOS differed from that dependent on PKC (Ser896) based on the differential distribution of these two proteins among the spinal cord laminae (regions). Thus the pnNOS-ir cells were mainly located in SDH (laminae I–II), which is also rich in sigma-1 receptors. However, most of the PKC-dependent pGluN1-ir cells were detected in the deeper dorsal horn (laminae III–IV and V–VI) in the present study. The most obvious explanation for this discrepancy is that NO, being a diffusible gas,

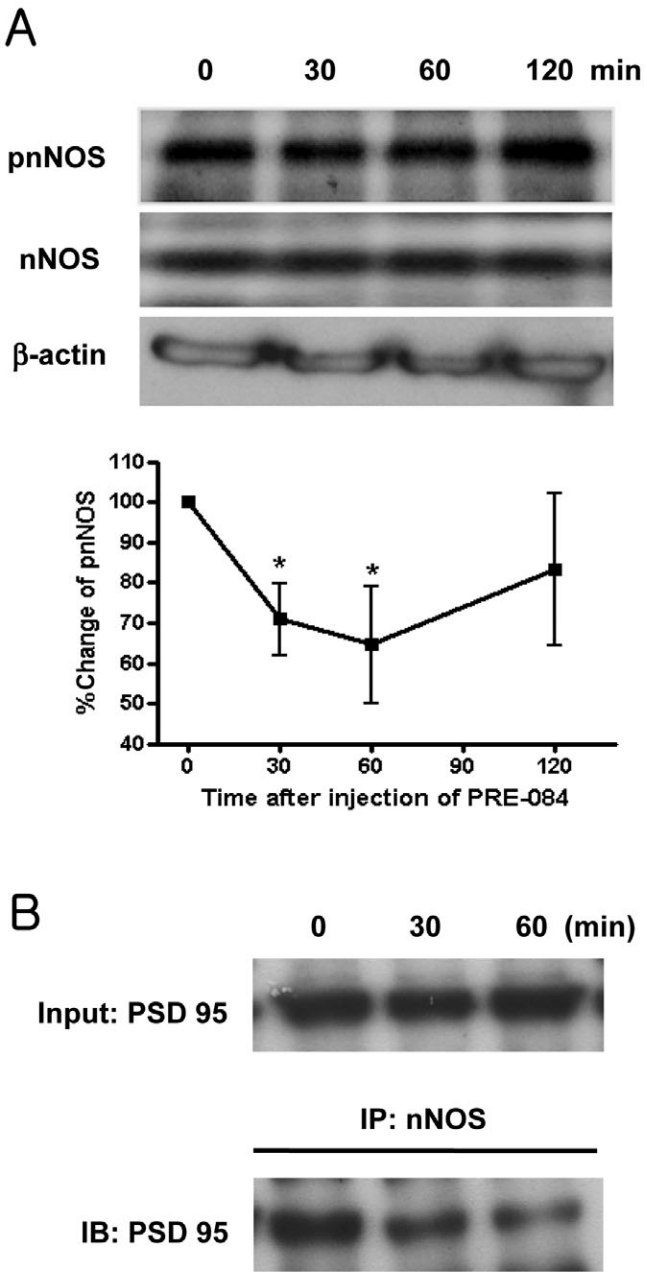


Figure 5

Changes in the phosphorylation (Ser847) and PSD95 binding of nNOS in spinal cord over time following intrathecal (i.t.) administration of PRE084 (PRE, 3 nmol). (A) Western blots illustrating the effect of i.t. injection of PRE084 on the ratio of pnNOS (Ser847) to nNOS expression in the spinal cord dorsal horn. The representative bands of pnNOS, nNOS and β-actin expression are presented in the upper portion of (A), and a graph depicting the change of the ratio of pnNOS (Ser847) to nNOS expression is shown in the lower portion of (A). A one-way ANOVA followed by Bonferroni's multiple comparison test was performed (*P < 0.05 as compared with non-treated value of normal mice). (B) A co-immunoprecipitation method was also used to determine the interaction of nNOS with PSD95 in spinal dorsal horn. Homogenates were immunoprecipitated (IP) with anti-nNOS, then blotted (IB) with anti-PSD95 antibody. Homogenates sampled at 0 (normal), 30, 60 min after PRE084 injection were examined. Input, 300μg of tissue lysates at each time point.

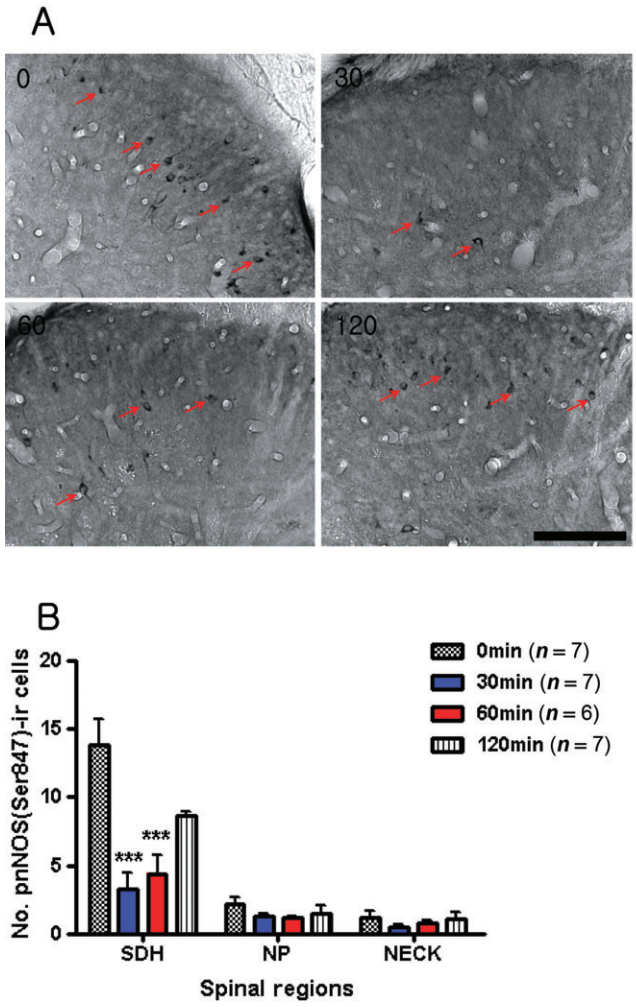


Figure 6

Representative photomicrographs (A) and graph (B) illustrating the effect of intrathecal (i.t.) injection of PRE084 (PRE, 3 nmol) on the number of pnNOS (Ser847)-immunoreative (ir) cells in the spinal cord dorsal horn. Red arrows indicate representative pnNOS-ir cells (A). Scale bar = 100 μm. The number of pnNOS-ir cells in the superficial dorsal horn (SDH, lamina I–II), in the nucleus proprius (NP, lamina III–IV) and in the neck region (NECK, lamina V–VI) of the spinal cord dorsal horn is depicted in (B). A one-way ANOVA followed by Bonferroni's multiple comparison test was performed (***P < 0.001 as compared with non-treated value of normal mice).

readily permeates cell membranes and thus it can diffuse from its site of production in laminae I and II to act on glia and/or other neurones in deeper laminae (Meller and Gebhart, 1993; Schmidtko *et al.*, 2009). Thus, even though NO is mainly produced in the SDH by sigma-1 receptor activation, it is reasonable that NMDA receptors (pGluN1) located on deep dorsal horn cells can be affected either directly or indirectly by NO produced more superficially. However, these concepts need to be confirmed by further investigation.

Finally, we also evaluated the effect of pretreatment with the sigma-1 receptor antagonist, BD1047, and the phosphatase CN inhibitor, CsA, and found that both of these

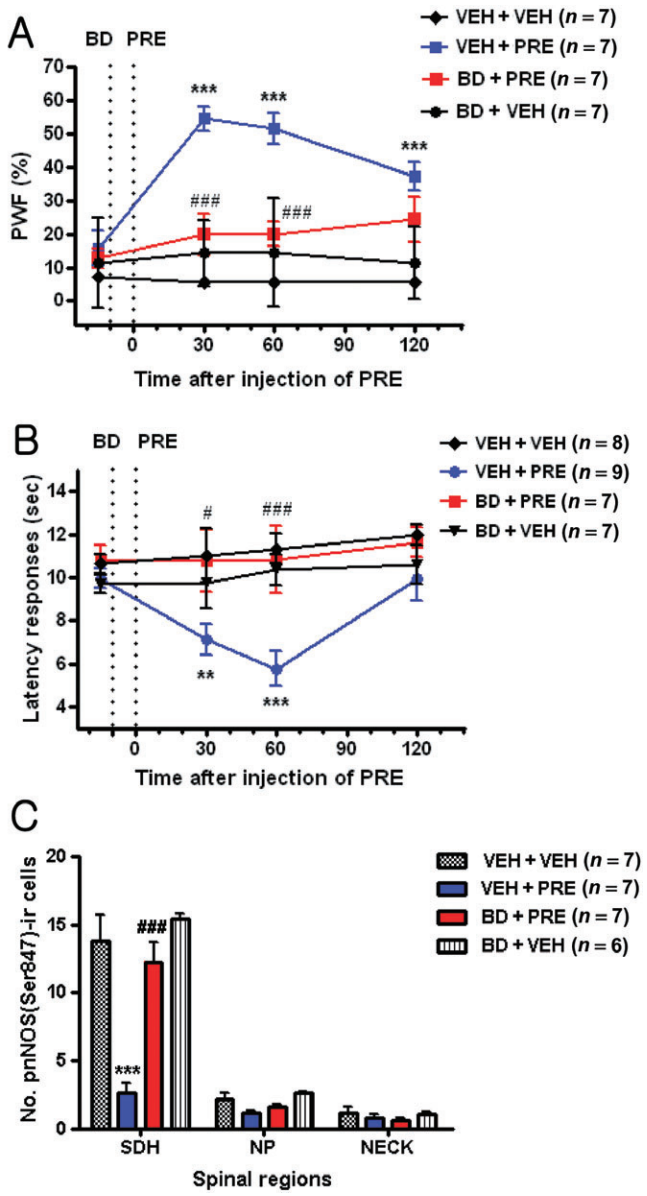


Figure 7

Effects of intrathecal (i.t.) administration of BD1047 (BD, 100 nmol), a sigma-1 receptor antagonist, on PRE084 (PRE; 3 nmol)-induced changes in the paw withdrawal frequency (PWF; %, A), in the latency responses (seconds, B) and in the number of pnNOS (Ser847)-immunoreactive (ir) cells in the spinal cord dorsal horn (C). BD was applied 10 min before PRE injection. Repeated measures two-way ANOVA or a one-way ANOVA followed by the Bonferroni's multiple comparison test was performed on the behavioural data and on the analysis of pnNOS-ir cells expression, respectively (**$P < 0.01$ and ***$P < 0.001$ as compared with those of the VEH + VEH group and #$P < 0.05$ and ###$P < 0.001$ as compared with those in the VEH + PRE group). SDH, superficial dorsal horn; NP, nucleus proprius; NECK, neck of dorsal horn.

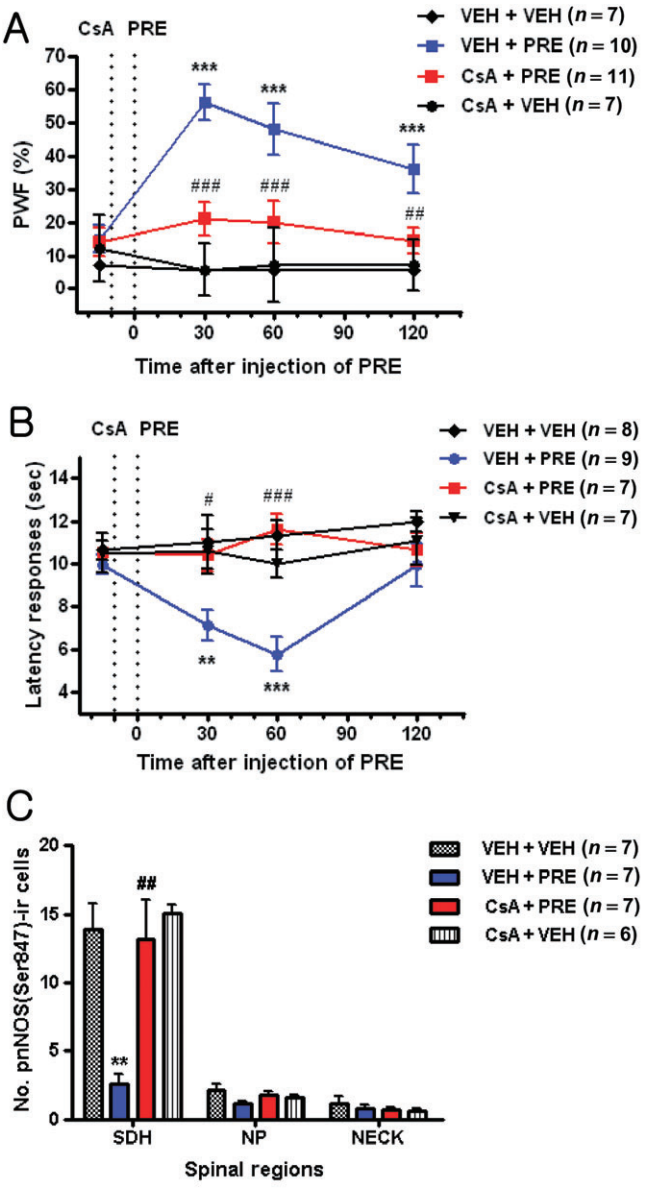


Figure 8

Effects of intrathecal (i.t.) administration of cyclosporin A (CsA; 20 nmol), a calcineurin inhibitor, on PRE084 (PRE; 3 nmol)-induced changes in the paw withdrawal frequency (PWF; %, A), in the latency responses (seconds, B) and in the number of pnNOS (Ser847)-immunoreactive (ir) cells (C). CsA was applied 10 min before PRE084 injection. A repeated measures two-way ANOVA or a one-way ANOVA followed by a Bonferroni's multiple comparison test was performed on the behavioural data and on the analysis of pnNOS-ir cell expression, respectively (**$P < 0.01$ and ***$P < 0.001$ as compared with those of the VEH + VEH group and #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ as compared with those in the VEH + PRE group). SDH, superficial dorsal horn; NP, nucleus proprius; NECK, neck of dorsal horn.

agents attenuated PRE084-induced mechanical and thermal hypersensitivity and prevented the sigma-1 receptor-induced decrease in the number of pnNOS-ir cells. The results obtained with BD1047 pretreatment confirmed that the PRE084-mediated pain induction was directly related to sigma-1 receptor activation, which in turn caused activation of an NO signalling cascade. In addition, the blocking effect of BD1047 pretreatment on the PRE084-induced increase in

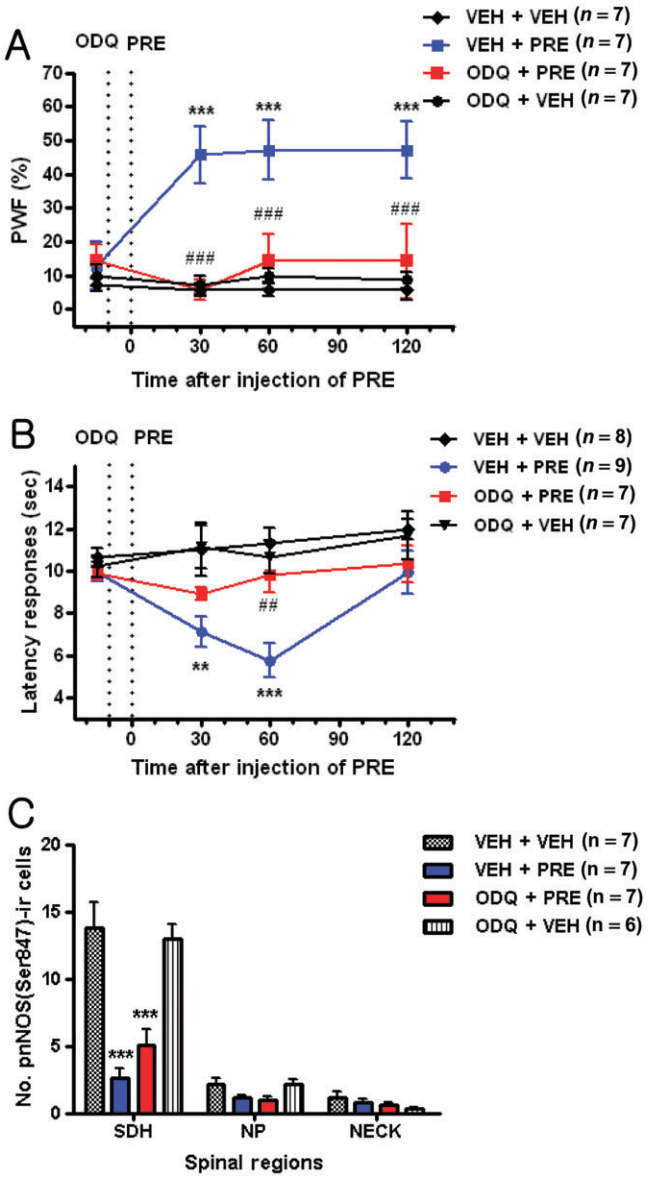


Figure 9

Effects of intrathecal (i.t.) administration of ODQ (300 pmol), a soluble guanylate cyclase inhibitor, on PRE084 (PRE, 3 nmol)-induced changes in the paw withdrawal frequency (PWF; %, A), in the latency responses (s, B) and in the number of pnNOS (Ser847)-immunoreactive (ir) cells in the spinal cord dorsal horn (C). ODQ was applied 10 min before PRE084 injection. A repeated measures two-way ANOVA or a one-way ANOVA followed by a Bonferroni's multiple comparison test was performed on the behavioural experimental data and on the analysis of pnNOS-ir cells, respectively (**$P < 0.01$ and ***$P < 0.001$ as compared with those of the VEH + VEH group and ##$P < 0.01$ and ###$P < 0.001$ as compared with those in the VEH + PRE group). SDH, superficial dorsal horn; NP, nucleus proprius; NECK, neck of dorsal horn.

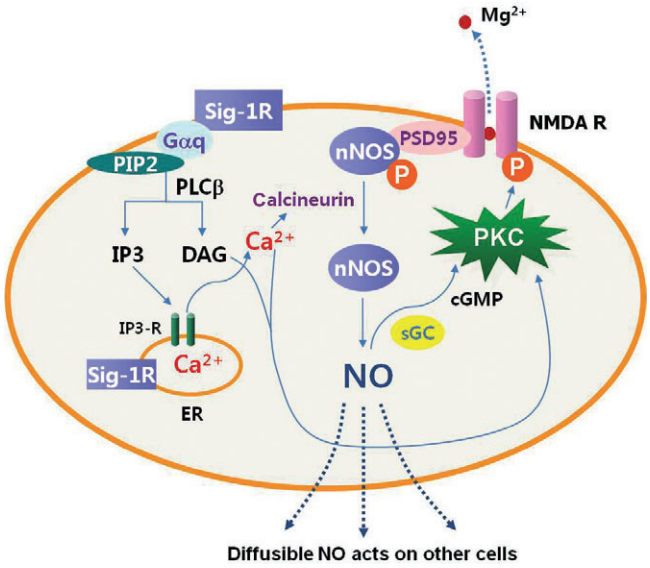


Figure 10

Schematic diagram that summarizes our proposed mechanism for how activation of the sigma-1 receptor (Sig-1R) in turn activates nitric oxide (NO), which then stimulates pGluN1(NMDA R) via a protein kinase C (PKC) mechanism to ultimately facilitate pain. The activation of the sigma-1 receptor stimulates PLC to hydrolyze PIP2 to produce diacyl glycerol (DAG) and IP3. IP3 then binds to IP3 receptors (IP3-R) in the endoplasmic reticulum to promote the efflux of Ca^{2+} to the cytoplasm. Increased cytosolic Ca^{2+} then influences calcineurin activity and via this mechanism reduces the phosphorylation of nNOS (i.e. resulting in an increase in nNOS activity). The NO generated from nNOS stimulates cGMP production via sGC, which in turn leads to an increase in PKC activity. The increase in cGMP is likely to produce the PKC activation, which induces the phosphorylation of the PKC-dependent NMDA receptor GluN1 subunit, resulting in the initiation of the pain facilitatory effect. In addition, the diffusible NO produced by sigma-1 receptor activation can also contribute to the pain facilitatory effect by diffusion from the cell to affect other cells (e.g. such as cells in the deep dorsal horn). Collectively our findings show that activation of the sigma-1 receptor leads to an increase in activated nNOS, which plays a key role in sigma-1 receptor-mediated mechanical and thermal hypersensitivity and PKC-dependent, but not PKA-dependent, pGluN1 expression.

pGluN1 expression and PKC activity has also been reported in our previous studies (Kim *et al.*, 2008; Roh *et al.*, 2008a; 2010). CN is the Ca^{2+}/calmodulin-dependent phosphatase of pnNOS, and activation of CN plays a critical role in nNOS activation (Zhou and Zhu, 2009). In this study, blocking CN activation by CsA prevented both NO signalling activation and PRE084-induced mechanical and thermal hypersensitivity. These results demonstrate that the NO signalling pathway via CN plays a key role in sigma-1 receptor-mediated pain induction. By contrast, the sGC inhibitor, ODQ, attenuated PRE084-mediated pain induction, but did not affect the sigma-1 receptor-induced decrease in the number of pnNOS-ir cells. As mentioned above one of the major NO signalling mechanisms in the spinal nociceptive process involves the activation of sGC and subsequent cGMP production (Tao and Johns, 2002). As inhibiting sGC with ODQ blocked the NO downstream pathway following pnNOS dephosphorylation, it is our contention that ODQ could not modify the sigma-1 receptor-induced decrease in pnNOS-ir cells.

In conclusion, in the present study it was demonstrated that spinal sigma-1 receptor-induced mechanical and

thermal hypersensitivity are mediated by an increase in activated nNOS (i.e. as evidenced by a significant decrease in pnNOS and/or by the decrease in the PSD95-PDZ domain binding form), which in turn causes an increase in NO-induced PKC-dependent, PKA-independent, pGluN1 expression (see Figure 10, a schematic diagram that summarizes the mechanism by which activation of the sigma-1 receptor stimulates NO signalling and affects PKC-dependent pGluN1 expression). Collectively these findings suggest that the increase in NO signalling that occurs via a sigma-1 receptor-mediated decrease in pnNOS plays an important role in spinal sigma-1 receptor-mediated sensitization.

Acknowledgements

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Conflicts of interest

None.

References

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR *et al.* (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 84: 757–767.

Chu YC, Guan Y, Skinner J, Raja SN, Johns RA, Tao YX (2005). Effect of genetic knockout or pharmacologic inhibition of neuronal nitric oxide synthase on complete Freund's adjuvant-induced persistent pain. Pain 119: 113–123.

Collier TL, Waterhouse RN, Kassiou M (2007). Imaging sigma receptors: applications in drug development. Curr Pharm Des 13: 51–72.

Duman EN, Kesim M, Kadioglu M, Ulku C, Kalyoncu NI, Yaris E (2006). Effect of gender on antinociceptive effect of paroxetine in hot plate test in mice. Prog Neuropsychopharmacol Biol Psychiatry 30: 292–296.

Garthwaite J (2008). Concepts of neural nitric oxide-mediated transmission. Eur J Neurosci 27: 2783–2802.

Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS (2005). Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol 6: 150–166.

Hylden JL, Wilcox GL (1980). Intrathecal morphine in mice: a new technique. Eur J Pharmacol 67: 313–316.

Karlsson J, Fong KS, Hansson MJ, Elmer E, Csiszar K, Keep MF (2004). Life span extension and reduced neuronal death after weekly intraventricular cyclosporin injections in the G93A transgenic mouse model of amyotrophic lateral sclerosis. J Neurosurg 101: 128–137.

Kim HW, Kwon YB, Roh DH, Yoon SY, Han HJ, Kim KW *et al.* (2006). Intrathecal treatment with sigma1 receptor antagonists reduces formalin-induced phosphorylation of NMDA receptor subunit 1 and the second phase of formalin test in mice. Br J Pharmacol 148: 490–498.

Kim HW, Roh DH, Yoon SY, Seo HS, Kwon YB, Han HJ *et al.* (2008). Activation of the spinal sigma-1 receptor enhances NMDA-induced pain via PKC- and PKA-dependent phosphorylation of the NR1 subunit in mice. Br J Pharmacol 154: 1125–1134.

Koesling D, Russwurm M, Mergia E, Mullershausen F, Friebe A (2004). Nitric oxide-sensitive guanylyl cyclase: structure and regulation. Neurochem Int 45: 813–819.

Kwon YB, Lee JD, Lee HJ, Han HJ, Mar WC, Kang SK *et al.* (2001). Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. Pain 90: 271–280.

Lee JH, Wilcox GL, Beitz AJ (1992). Nitric oxide mediates Fos expression in the spinal cord induced by mechanical noxious stimulation. Neuroreport 3: 841–844.

Levy D, Zochodne DW (2004). NO pain: potential roles of nitric oxide in neuropathic pain. Pain Pract 4: 11–18.

Maurice T, Su TP (2009). The pharmacology of sigma-1 receptors. Pharmacol Ther 124: 195–206.

Meller ST, Gebhart GF (1993). Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 52: 127–136.

Milano J, Oliveira SM, Rossato MF, Sauzem PD, Machado P, Beck P *et al.* (2008). Antinociceptive effect of novel trihalomethyl-substituted pyrazoline methyl esters in formalin and hot-plate tests in mice. Eur J Pharmacol 581: 86–96.

Miyamoto T, Dubin AE, Petrus MJ, Patapoutian A (2009). TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. PLoS ONE 4: e7596.

National Institute of Health (NIH) (1985). *Guide for the Care and Use of Laboratory Animals*. DHEW Publication (NIH), revised, Office of Science and Health Reports, DRR/NIH: Bethesda, MD.

Osuka K, Watanabe Y, Usuda N, Nakazawa A, Fukunaga K, Miyamoto E *et al.* (2002). Phosphorylation of neuronal nitric oxide synthase at Ser847 by CaM-KII in the hippocampus of rat brain after transient forebrain ischemia. J Cereb Blood Flow Metab 22: 1098–1106.

Osuka K, Watanabe Y, Usuda N, Atsuzawa K, Aoshima C, Yamauchi K *et al.* (2007). Phosphorylation of neuronal nitric oxide synthase at Ser847 in the nucleus intermediolateralis after spinal cord injury in mice. Neuroscience 145: 241–247.

Palmer RM, Ashton DS, Moncada S (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333: 664–666.

Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC *et al.* (1999). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. Circ Res 84: 587–604.

de la Puente B, Nadal X, Portillo-Salido E, Sanchez-Arroyos R, Ovalle S, Palacios G *et al.* (2009). Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. Pain 145: 294–303.

Rameau GA, Chiu LY, Ziff EB (2004). Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. J Biol Chem 279: 14307–14314.

Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW *et al*. (2008a). Intrathecal administration of sigma-1 receptor agonists facilitates nociception: involvement of a protein kinase C-dependent pathway. J Neurosci Res 86: 3644–3654.

Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW *et al*. (2008b). Intrathecal injection of the sigma(1) receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. Anesthesiology 109: 879–889.

Roh DH, Yoon SY, Seo HS, Kang SY, Moon JY, Song S *et al*. (2010). Sigma-1 receptor-induced increase in murine spinal NR1 phosphorylation is mediated by the PKCalpha and varepsilon, but not the PKCzeta, isoforms. Neurosci Lett 477: 95–99.

Schmidtko A, Gao W, Konig P, Heine S, Motterlini R, Ruth P *et al*. (2008). cGMP produced by NO-sensitive guanylyl cyclase essentially contributes to inflammatory and neuropathic pain by using targets different from cGMP-dependent protein kinase I. J Neurosci 28: 8568–8576.

Schmidtko A, Tegeder I, Geisslinger G (2009). No NO, no pain? The role of nitric oxide and cGMP in spinal pain processing. Trends Neurosci 32: 339–346.

Tanabe M, Nagatani Y, Saitoh K, Takasu K, Ono H (2009). Pharmacological assessments of nitric oxide synthase isoforms and downstream diversity of NO signaling in the maintenance of thermal and mechanical hypersensitivity after peripheral nerve injury in mice. Neuropharmacology 56: 702–708.

Tao YX, Johns RA (2002). Activation and up-regulation of spinal cord nitric oxide receptor, soluble guanylate cyclase, after formalin injection into the rat hind paw. Neuroscience 112: 439–446.

Xu L, Mabuchi T, Katano T, Matsumura S, Okuda-Ashitaka E, Sakimura K *et al*. (2007). Nitric oxide (NO) serves as a retrograde messenger to activate neuronal NO synthase in the spinal cord via NMDA receptors. Nitric Oxide 17: 18–24.

Yonezawa T, Kurata R, Kimura M, Inoko H (2009). PKC delta and epsilon in drug targeting and therapeutics. Recent Pat DNA Gene Seq 3: 96–101.

Yoon SY, Kwon YB, Kim HW, Roh DH, Seo HS, Han HJ *et al*. (2008). Bee venom injection produces a peripheral anti-inflammatory effect by activation of a nitric oxide-dependent spinocoeruleus pathway. Neurosci Lett 430: 163–168.

Zhou C, Li C, Yu HM, Zhang F, Han D, Zhang GY (2008). Neuroprotection of gamma-aminobutyric acid receptor agonists via enhancing neuronal nitric oxide synthase (Ser847) phosphorylation through increased neuronal nitric oxide synthase and PSD95 interaction and inhibited protein phosphatase activity in cerebral ischemia. J Neurosci Res 86: 2973–2983.

Zhou L, Zhu DY (2009). Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric Oxide 20: 223–230.