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RESEARCH PAPER

Disruption of nNOS-PSD95 protein–protein interaction inhibits acute thermal hyperalgesia and chronic mechanical allodynia in rodents

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Background and purpose: Post-synaptic density protein 95 (PSD95) contains three PSD95/Dosophilia disc large/ZO-1 homology domains and links neuronal nitric oxide synthase (nNOS) with the N-methyl-D-aspartic acid (NMDA) receptor. This report assesses the effects of disruption of the protein–protein interaction between nNOS and PSD95 on pain sensitivity in rodent models of hyperalgesia and neuropathic pain.
Experimental approach: We generated two molecules that interfered with the nNOS–PSD95 interaction: IC87201, a small molecule inhibitor; and tat-nNOS (residues 1–299), a cell permeable fusion protein containing the PSD95 binding domain of nNOS. We then characterized these inhibitors using *in vitro* and *in vivo* models of acute hyperalgesia and chronic allodynia, both of which are thought to require nNOS activation.
Key results: IC87201 and tat-nNOS (1–299) inhibited the *in vitro* binding of nNOS with PSD95, without inhibiting nNOS catalytic activity. Both inhibitors also blocked NMDA-induced 3′,5′-cyclic guanosine monophosphate (cGMP) production in primary hippocampal cultures. Intrathecal administration of either inhibitor potently reversed NMDA-induced thermal hyperalgesia in mice. At anti-hyperalgesic doses, there was no effect on acute pain thresholds or motor coordination. Intrathecal administration of IC87201 and tat-nNOS also reversed mechanical allodynia induced by chronic constriction of the sciatic nerve.
Conclusions and implications: nNOS–PSD95 interaction is important in maintaining hypersensitivity in acute and chronic pain. Disruption of the nNOS–PSD95 interaction provides a novel approach to obtain selective anti-hyperalgesic compounds.
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Keywords: glutamate; neuronal nitric oxide synthase; post-synaptic density protein 95; hyperalgesia; neuropathic pain

Abbreviations: 7-NI, 7-nitroindazole; ANOVA, analysis of variance; BH_4, tetrahydrobiopterin; BSA, bovine serum albumin; CCI, chronic constriction injury; cGMP, 3′,5′-cyclic guanosine monophosphate; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol tetraacetic acid; GST, glutathione S-transferase; L-NAME, N^G-nitro-L-arginine methyl ester; MED, minimum effective dose; NMDA, N-methyl-D-aspartic acid; NOS, nitric oxide synthase; nNOS, neuronal NOS; PBS, phosphate buffered saline; PDZ, PSD95/Drosophila disc large/ZO-1 homology; PSD95, post-synaptic density protein 95; SNL, spinal nerve ligation

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Introduction

Central sensitization is important in the development and maintenance of chronic pain (Bennett, 2000). One of the key mediators of central sensitization is glutamate, the principal excitatory neurotransmitter in the central nervous system (Collingridge and Bliss, 1995; Nicoll and Malenka, 1999; Bennett, 2000). Glutamate is responsible for many forms of neuronal plasticity, a process proposed for the development and maintenance of nerve injury-evoked hyperalgesia and allodynia, which are central features of chronic neuropathic pain. NMDA receptors, one of the three families of ionotropic

glutamate receptors (Dingledine *et al.*, 1999), have been implicated in several chronic pain states as well as in central sensitization (Fisher *et al.*, 2000; Chizh and Headley, 2005).

As NMDA receptors are involved in many pathological processes, NMDA receptor antagonists have been an attractive drug development target. However, their therapeutic potential is limited by adverse events such as memory impairment, psychotomimetic effects and ataxia (Parsons, 2001; Chizh and Headley, 2005). This is not surprising because NMDA receptors are widely distributed in the nervous system where they are involved in many processes including several forms of learning and memory.

One consequence of NMDA receptor activation is the entry of calcium, which binds to calmodulin to activate downstream effectors including neuronal nitric oxide synthase (nNOS). The subsequent overproduction of nitric oxide (NO) is thought to promote hyperalgesia (Ji and Strichartz, 2004). At physiological levels, NO plays a key role as a second messenger. At elevated levels, the toxicity of NO dominates (Bredt and Snyder, 1994). Because of its non-enzymatic degradation and brief half-life, NO levels are primarily regulated by its synthetic enzyme, NOS. There are three isoforms of NOS (Alderton *et al.*, 2001), endothelial NOS, inducible NOS and nNOS, and the latter two are thought to be involved in spinal mechanisms underlying the development of persistent nociceptive signalling. A role for NOS in exaggerated nociceptive signalling was first observed in a model of acute thermal hyperalgesia evoked by intrathecal (i.t.) injection of NMDA in mice (Kitto *et al.*, 1992). Pretreatment with the non-selective NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), prevented the development of NMDA-evoked thermal hyperalgesia in this model. L-NAME also prevented the development and/or maintenance of hyperalgesia evoked by both the chronic constriction injury (CCI) (Meller *et al.*, 1992; Thomas *et al.*, 1996) and spinal nerve ligation (SNL) (Yoon *et al.*, 1998) models, confirming a role for NOS in these widely used preclinical models of neuropathic pain. Subsequent studies extended these investigations to show a specific role for the nNOS isoform using the nNOS selective inhibitor 7-nitroindazole (7-NI) to reverse hyperalgesia evoked by i.t. NMDA (Roberts *et al.*, 2005), CCI (Naik *et al.*, 2006) and SNL (Guan *et al.*, 2007) models. Guan *et al.* (2007) further showed that mice with a targeted disruption of the gene for the nNOS isoform failed to develop mechanical allodynia or hyperalgesia following SNL. Together, these results indicate that three distinct models of NMDA receptor-mediated hyperalgesia and/or allodynia also require the neuronal isoform of NOS.

Post-synaptic density protein 95 (PSD95) uses a combination of three PSD95/Drosophila disc large/ZO-1 homology (PDZ) domains to recruit proteins, including nNOS to the NMDA receptor (Sattler *et al.*, 1999). This close positioning of nNOS to the NMDA receptor allows for the effective activation of nNOS by calcium entering through the receptor. Overactivation of the NMDA receptor results in high levels of NO that may be toxic (Aarts and Tymianski, 2003). Of the three NOS isoforms, nNOS is unique in that it contains an N-terminal PSD95-binding domain, which is required for functional coupling of nNOS to the NMDA receptor–PSD95 complex (Brenman *et al.*, 1996; Kornau and Seeburg, 1997; Christopherson *et al.*, 1999; Tochio *et al.*, 1999; 2000a,b). Suppression of PSD95 expression with antisense oligonucleotides decreased NMDA-induced NO production and NMDA-mediated excitoxicity in cultured neurons without affecting NMDA receptor channel properties (Sattler *et al.*, 1999). Similar results were obtained with interfering RNA (Cui *et al.*, 2007). In rats, antisense to PSD95 decreased NMDA-induced thermal hyperalgesia (Tao *et al.*, 2000) and delayed the development of neuropathic pain (Tao *et al.*, 2001). Mice expressing a mutant form of PSD95 that could not associate with the NMDA receptor (Migaud *et al.*, 1998) also failed to develop hyperalgesia and allodynia after CCI (Garry *et al.*, 2003). These results suggest that PSD95 is critical for NMDA-nNOS-mediated pain hypersensitivity.

A novel approach to selectively inhibit the NMDA–PSD95–nNOS signalling pathway is to disrupt the nNOS–PSD95 interaction. This would allow for specific inhibition of the NMDA-nNOS signalling pathway, without blocking NMDA-dependent, but nNOS-independent signalling, thereby sparing unwanted effects on many other important physiological processes mediated by the NMDA receptors. Similar approaches have been used to disrupt the interaction between nNOS and PSD95 in p38-mediated stress-induced cell death (Cao *et al.*, 2005) and the interaction between NMDA receptors and PSD95 in animals with ischaemic brain damage (Aarts *et al.*, 2002; Sun *et al.*, 2008). In this report, we use inhibitors of the nNOS–PSD95 protein–protein interaction to demonstrate the importance of nNOS targeting in mediating nociceptive signalling in two rodent models of hyperalgesia and allodynia and, thus, identify a novel approach to treat chronic pain.

Methods

Generation of fusion proteins

nNOS (a.a. 1–299, encoding the PSD95 binding domain) was generated by inserting human nNOS residues 1–299 into pGEX 4T3 such that the clone was in frame with the glutathione S-transferase (GST) coding sequence of the vector. This 'GST-nNOS' was expressed in bacteria, and purified using glutathione Sepharose chromatography and thrombin cleavage, eluting purified nNOS 1–299 protein. This nNOS (1–299) was used in the *in vitro* binding assay. The protein sequence for human nNOS (1–299) is 94% and 96% homologous to mouse and rat nNOS (1–299) respectively.

Tat-nNOS (1–299) fusion protein was generated by insertion of human nNOS residues 1–299 into a pRSET-B vector containing the coding sequence for the protein-transduction domain (YGRKKRRQRRR) of HIV-1 Tat protein. This tat-nNOS fusion contained a Tat-sequence and either a 6× or 10× His-tag at its N-terminal. Tat-nNOS fusion protein was expressed in bacteria, purified under denaturing conditions on a nickel-nitrilotriacetic acid (NTA) column and dialyzed against 1× calcium and magnesium-free phosphate buffered saline (PBS) before use. For a negative control, a non-transducing tat-nNOS containing the sequence (KALGISYGRKK) of Tat protein and the same 1–299 residues of nNOS was used.

PSD95 containing PDZ domains 1–3 (residues 1–435, based on the human PSD95 sequence) was subcloned into a biotin expression plasmid such that the coding sequence was in frame with the biotin acceptor peptide. The fusion protein

was expressed in bacteria in the presence of biotin and purified using streptavidin affinity chromatography. The purified protein contained biotin at its internal biotin acceptor site and is referred to as biotinylated PSD95. This was used in the *in vitro* binding assay. PDZ domain two of PSD95 is mainly responsible for the binding PSD95 to nNOS (Cho *et al*., 1992) and this protein sequence is 100% identical between human, rat and mouse.

In vitro nNOS–PSD95 interaction assay

Recombinant nNOS (a.a. 1–299, 5 $\mu g \cdot mL^{-1}$), cleaved from GST-nNOS, was used to coat wells of an Immulon 96-well plate. After blocking non-specific sites with SEA block (Pierce) and further washing, biotinylated PSD95 (12.5 nM) was added as 'ligand' and binding continued for 2 h at room temperature before the reaction was terminated by repeated washing with PBS containing 0.05% Tween 20. The biotinylated PSD95–nNOS complex was detected by streptavidin–europium (Perkin-Elmer, Waltham, MA, USA). After release of the europium by an enhancement solution (Perkin-Elmer), the increased fluorescence was measured using a DELFIA research fluorimeter (Perkin-Elmer). Using this *in vitro* binding assay, a high throughput screen was carried out to identify small molecule inhibitors which can disrupt the protein–protein interaction between nNOS and PSD95. Inhibitors were then tested for their ability to block the NMDA-induced increase in cGMP production (an indirect measurement of NO production) in neuronal cultures. One lead compound was identified to have efficacy in cell-based assays and was modified to improve stability. This modified small molecule, IC87201, 2-((1H-benzo[d][1,2,3]trizol-5-ylamino)methyl)-4-6-dichlorophenol (Figure 1), was further characterized.

nNOS enzymatic assay

nNOS was partially purified from frozen rat brain (Pel-Freez, Rogers, AR, USA) supernatant using 2′,5′-ADP sepharose and calmodulin-sepharose chromatography (both from Pharmacia Biotech, Piscataway, NJ, USA), according to Schmidt *et al*. (1991). The final pooled fractions, in 50 mM Tris, pH 7.5, 2 mM dithiothreitol (DTT), 1 M NaCl, 10% glycerol and 5 mM ethylene glycol tetraacetic acid (EGTA), were collected, concentrated and frozen. nNOS enzymatic activity was measured by the conversion of oxyhaemoglobin to methaemoglobin by NO essentially as described in Dawson and Knowles (1998). All buffers, inhibitors and equipment were prewarmed to 37°C prior to assay. The final concentration of the components in the assay were 50 mM HEPES, pH 7.4, 100 μM DTT, 1 mM $CaCl_2$, 5 μM oxyhaemoglobin, 12 μM tetrahydrobiopterin (BH4), 120 μM NADPH, 1 μM FMN, 1 μM FAD and 0.1 μM calmodulin. The reaction was initiated by the addition of partially purified rat brain nNOS. After quick mixing, the reaction absorbance was continuously measured at 405 nM and 420 nM for 30–60 min (at 10–20 s intervals) on a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA, USA). L-N^G-monomethyl arginine citrate was used as a positive control.

NMDA-induced increase in cGMP in primary rat hippocampal neurons

Neonatal rat hippocampal cultures were prepared according to Brewer (1997). Cells were cultured for 14–21 days before testing. NMDA (100 μM final) increased cGMP (measured by a cGMP-RIA kit from Perkin-Elmer) within 2–15 min of addition. The NMDA receptor antagonist, MK-801, and a NOS catalytic inhibitor, L-NAME, were used as positive controls.

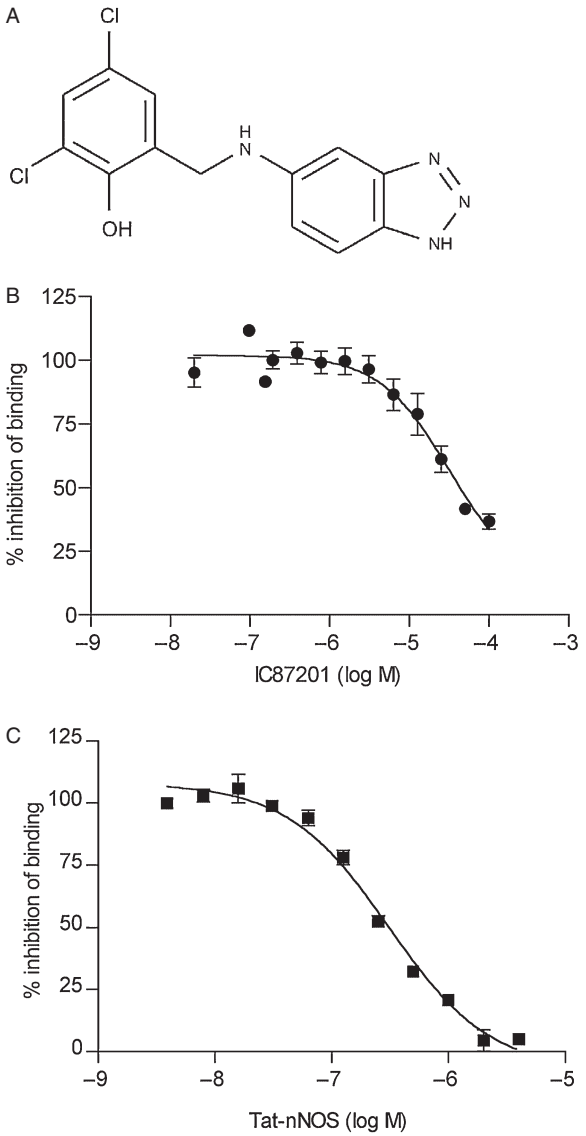

Figure 1 (A) Structure of IC87201. (B) and (C) *In vitro* binding assays. IC87201 (B) and tat-nNOS 1–299 (C) dose-dependently inhibited the interaction between nNOS (1–299) and PSD95 in a plate-binding assay, where nNOS was coated on a 96-well plate and biotinylated PSD95 added as a ligand. IC_{50} for IC87201 is 31 μM (n = 5) and for tat-nNOS is 0.3 μM (representative of eight experiments). See the text for statistical analysis. nNOS, neuronal nitric oxide synthase; PSD95, post-synaptic density protein 95.

In vivo studies

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota or the Institutional Animal Care and Use Committee of Brown University. Outbred Institute of Cancer Research (ICR-CD1, Harlan Madison, WI, USA) male mice weighing 21–24 g were used in the NMDA-induced

hyperalgesia experiments. Adult male Sprague–Dawley rats (Charles River, Boston, MA, USA; 350–450 g) were used in the neuropathic pain experiments. In both cases, animals were housed in a temperature-controlled and humidity-controlled environment, and maintained on a 12 h light/dark cycle, with free access to food and water.

Compound preparation testing
IC87201, 2-((1H-benzo[d][1,2,3]triazol-5-ylamino)methyl)-4,6-dichlorophenol, was synthesized by standard reductive amination of appropriate amines and aldehydes. For *in vitro* assays, IC87201 was prepared in 100% DMSO, then diluted into PBS and 0.1% bovine serum albumin (BSA). For cell-based assays, IC87201 was prepared in 100% DMSO and then diluted into control saline solution (120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM Tris-HCl, 15 mM glucose, pH 7.5). For mouse experiments, IC87201 was prepared from a stock solution of 20 mM in 50% DMSO/50% 0.9% saline. This stock was then diluted to appropriate concentrations with a final DMSO concentration of 5% or less. Injection volume was 5 μL for i.t. administration. For i.p. administration, the injection volume was 100 μL. For rat models, IC87201 was dissolved in 20% DMSO in PBS, then 10 μL was administered through an i.t. catheter. Tat-nNOS and nt-tat-nNOS were purified as described above and dialyzed into 0.9% saline or PBS. In all cases, studies were vehicle matched.

Tail flick analgesia
Antinociception was tested by tail immersion in warm water (52.5°C), and the latency to rapid tail flick was measured in seconds (Fairbanks *et al.*, 2000).

Intrathecal injections in a mouse thermal hyperalgesia model
NMDA (Sigma, St. Louis, MO, USA) was given intrathecally in conscious mice according to the method of Hylden and Wilcox (1980), as described in detail by Fairbanks (2003). Briefly, the pelvic girdle (ileac crest) of the mouse was gripped firmly by the thumb and forefinger of the injector's non-dominant hand. The skin above the ileac crest was pulled tautly to create a horizontal plane where the needle was inserted. The needle was a 30 gauge, 0.5 inch sterile disposable needle connected to a 50 μL Luer-hub Hamilton syringe. All injections were delivered in 5 μL of vehicle, a frequently used volume in pharmacodynamic studies of intrathecally delivered agents in mice. All doses are expressed as total dose in moles.

NMDA-induced nociceptive behavioural responses
Intrathecal administration of NMDA (0.3 nmol) produced scratching and biting responses in the first minute after injection (Aanonsen and Wilcox, 1987). This NMDA-induced scratching behaviour is blocked by NMDA receptor antagonists, but not by NOS catalytic inhibitors (Roberts *et al.*, 2005), thus, it appears to be NO independent.

NMDA-induced thermal hyperalgesia
Intrathecal administration of NMDA (0.3 nmol) in mice also produced thermal hyperalgesia, which was measured by warm water (49°C) immersion test (adapted from the radiant heat method of Kitto *et al.*, 1992) 5 min after NMDA injection. The animals showed a decrease in tail flick latency (1–2 s) in response to applied noxious heat (49°C) relative to their baseline and also relative to saline-injected controls. This test was conducted immediately after and in the same animals in which the NMDA nociceptive scratching and biting behaviours were counted. Inhibitors were either co-administered intrathecally with NMDA or administered prior to NMDA injection (as indicated in results). In dose response experiments using i.t. administration, IC87201 or tat-nNOS was co-administered (i.t.) with NMDA, then nociceptive behaviours (immediately after administration) and tail flick latencies (5 min after administration) were measured in the same mice. In most experiments, motor coordination using the rotarod was also measured. Similar measurements were made for i.p. administration except that the inhibitors were administered i.p. 1 h before i.t. administration of NMDA. In time course experiments, inhibitors were administered (i.t. or i.p.) for the indicated time points before i.t. administration of NMDA. Thus, in the case of time course studies for i.t. administration of IC87201, each mouse was dosed i.t. twice, first with the inhibitor, then with NMDA. Data are expressed as either tail flick latencies (in seconds) or as the average percent inhibition ± SEM, calculated by the formula: $[(\text{control}\Delta - \text{experimental}\Delta)/\text{control}\Delta] \times 100\%$. All studies were vehicle matched. For IC87201, the vehicle was 0.9% saline/5% DMSO, and for tat-nNOS, the vehicle was 0.9% saline. MK-801 and the nNOS catalytic inhibitor, 7-NI, were used as positive controls.

Rotarod assay of sedation/motor impairment
After two training sessions, mice were placed for 300 s on an accelerating (4–40 rpm) rotarod (Ugo Basile, Varese, Italy). We measured the latency to fall before and after delivery of buffer, IC87201, tat-nNOS or control tat-nNOS. Data are presented as the time (seconds) the mice stayed on the rotarod (with 300 s as the maximum) or by the formula: % motor impairment = (pre-drug latency – post-drug latency)/(pre-drug latency × 100%) (Fairbanks *et al.*, 2000). In this latter case, mice that walked for 300 s would have a motor impairment of 0%.

Spinal catheter implantation and the CCI model
Adult male Sprague–Dawley rats (Charles Rivers; 350–450 g) were anaesthetized with ketamine and medetomidine (75 $mg \cdot kg^{-1}$, 25 $mg \cdot kg^{-1}$, i.p.). The spinal catheter was implanted using the method of Yaksh and Rudy (1976) so that the distal end of the catheter extended to the lumbar enlargement. CCI was then performed on the left sciatic nerve trunk as described by Bennett and Xie (1988). Animals were given atipamezole (25 $mg \cdot kg^{-1}$, i.p.) post-surgery and monitored until recovery from anaesthesia. Animals were tested for signs of motor impairment 1 day post-surgery and those with impaired motor function were excluded from further experimentation. Spinal catheters were flushed with 10 μL of sterile saline each post-operative day with the exception of drug-testing day.

Measurement of mechanical allodynia in CCI rats
Mechanical allodynia was assessed with calibrated von Frey filaments. Rats were tested pre-surgery and every 1–2 days for up to 14 post-operative days to monitor the development of neuropathic pain. Following a 20 min acclimation period, von Frey filaments of increasing force were applied to the plantar surface of the hindpaw and held for 8 s or until the animal withdrew its paw. Each testing set was conducted 5 min apart, alternating between the left and right paws. Mechanical allodynia typically developed between post-operative days 1–7, as indicated by a significant difference between injured and uninjured paw. Upon detection of mechanical allodynia, drug testing was performed the following day. Drug or vehicle (10 μL) followed by saline (10 μL) was infused through the spinal catheter. Testing commenced immediately following injection and continued at 5 min intervals for at least 2 h. Twenty-four hours later, all animals were reassessed for mechanical allodynia both at baseline and following infusion of 15 μg of morphine sulphate as a positive control for proper placement of the catheter. At the conclusion of morphine testing, the animals were transcardially perfused with formalin (4%). A saturated solution of fast green dye (10 μL) was infused through the spinal catheter to mark the distal end of the catheter. Animals in which the catheter tip deviated from the lumbar enlargement by more than 1 cm or were unresponsive to i.t. morphine administration were excluded from the study. Repeated measures analysis of variance was conducted with the Huynh–Feldt correction applied where applicable (BMDP Statistical Software, Los Angeles, CA, USA). $P < 0.05$ was considered statistically significant. The experimenter was unaware of the treatments given to the animals.

Pharmacokinetic studies
IC87201 was dissolved in a mixture containing DMSO and saline (50:50, v/v) to obtain a 6.0 mg·mL^{-1} stock solution. The solution was diluted further with saline just before dosing to obtain the desired administration levels. Similar rat and mouse strains were used as the *in vivo* studies. After i.p. injection (100–200 μL) into rats, 200 μL aliquots of blood samples were collected from 0 h to 24 h and the plasma processed and analysed by LC/MS/MS. For mice, blood was collected by cardiac puncture at the same time points as the rat studies and processed and analysed similarly. The results were analysed by non-compartmental analysis using WinNonlin (Pharsight, Mountain View, CA, USA).

Data analysis
Values are expressed as means, and variability as SEM or as 95% confidence limits. In experiments with three or more doses that are statistically significant from control (vehicle control or zero time point; i.e. there is dose dependency), IC_{50} or EC_{50} values were calculated by non-linear regression analysis using the equation of a sigmoid concentration-response curve (GraphPad Prism). Depending on the experiment, statistical analysis is either paired *t*-test or ANOVA followed by Dunnett's or Bonferroni post-tests, using either vehicle treatment or pretreatment group as the comparator. $P < 0.05$ was considered statistically significant.

Results

IC87201 and tat-nNOS blocked the interaction between nNOS and PSD95 in vitro
A high throughput screen of 150 000 small molecules from the ICOS chemical library yielded several inhibitors that disrupted the protein–protein interaction of nNOS–PSD95 in an *in vitro* binding assay. One of these compounds was further modified to improve stability. The resulting compound, IC87201, 2-[(1H-benzotriazol-5-ylamino)-methyl]-4,6-dichloro-phenol (Figure 1A), showed dose-dependent inhibition of nNOS–PSD95 binding (Figure 1B) with an IC_{50} of 31 μM (95% confidence interval 25–38 μM, $n = 5$). IC87201 had no effect on an unrelated interaction between PSD95 and a PSD95 binding protein, cypin (data not shown). Tat-nNOS, containing the PSD95 binding domain of nNOS, also inhibited the interaction between nNOS–PSD95 (Figure 1C) with an IC_{50} of 0.3 μM (95% confidence interval 0.2–0.5 μM; $n = 8$). These two inhibitors were used to determine the effects of disruption of the nNOS–PSD95 interaction in both cell-based and animal studies.

IC87201 and tat-nNOS attenuated NMDA-induced cGMP production in primary neuronal cultures
NMDA receptor stimulation in primary hippocampal neurons activates nNOS. The resultant increase in NO stimulates guanylate cyclase to form cGMP. Thus, cGMP levels can serve as a reasonable indicator of NO activation. The NMDA receptor antagonist, MK-801, or the non-selective NOS inhibitor, L-NAME, both blocked NMDA-induced increase in cGMP production in hippocampal cultures, confirming that cGMP induction was dependent on the NMDA receptor activation of nNOS (Dawson *et al*., 1991). In this study, both IC87201 (Figure 2A) and tat-nNOS (Figure 2B) dose-dependently inhibited NMDA-induced cGMP production with IC_{50}'s of 2.7 μM (95% confidence interval 2.1–3.4 μM; $n = 14$–20) and 2.5 μM (95% confidence interval 0.4–18 μM; $n = 5$) respectively. IC87201 had no effect on cell viability at the concentrations tested (data not shown). When an NO donor, sodium nitroprusside, was used to bypass the NMDA receptor to activate guanylate cyclase directly, IC87201 had no effect on cGMP levels (98.7% of control at 5 μM, $n = 3$). Furthermore, IC87201, at 40 μM, did not inhibit nNOS enzymatic activity *in vitro* (92% of control). Together, these results suggest that the inhibitory effects of IC87201 were not due to catalytic inhibition of NOS and that IC87201 acts upstream of nNOS.

The small molecule inhibitor, IC87201, and tat-nNOS inhibited NMDA-induced thermal hyperalgesia in mice
As the NMDA receptor PSD95–nNOS pathway is involved in thermal hyperalgesia, we assessed whether inhibitors of nNOS–PSD95 protein–protein interaction would disrupt NMDA-induced hyperalgesia. Intrathecal administration of 0.3 nmol NMDA in mice induces thermal hypersensitivity

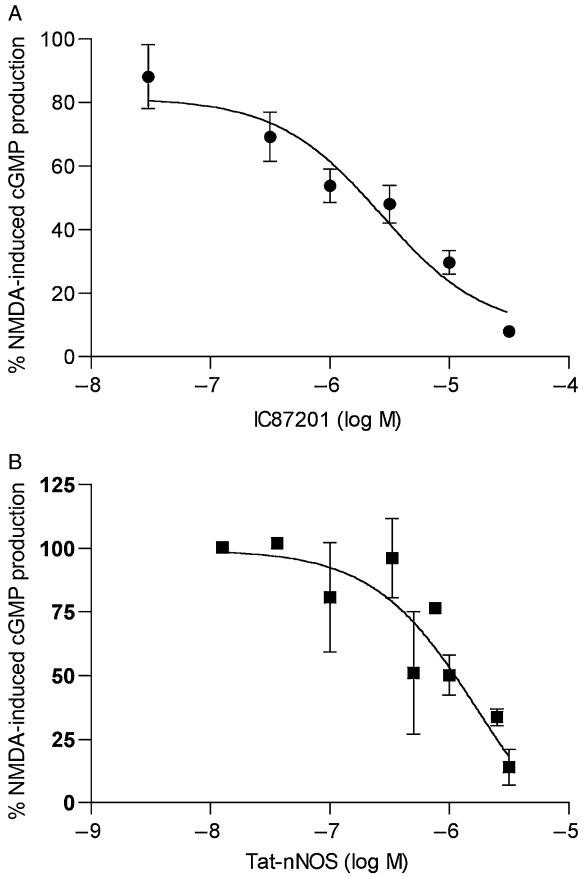

Figure 2 IC87201 (A) and tat-nNOS (B) attenuate the NMDA-induced increase of cGMP in primary cultured hippocampal neurons. Both inhibitors dose-dependently attenuated NMDA-induced increases in cGMP, an indirect measurement of nitric oxide production. The IC_{50} values for IC87201 (n = 14–20) and tat-nNOS are 2.7 μM and 2.5 μM (n = 5) respectively. cGMP, 3′,5′-cyclic guanosine monophosphate; NMDA, N-methyl-D-aspartic acid; nNOS, neuronal nitric oxide synthase.

(measured by tail flick latency to warm water) that requires nNOS activation (Roberts *et al.*, 2005). Tail flick latency was significantly reduced following NMDA (0.3 nmol) delivery (Figure 3A; P = 0.001 in a paired t-test between baseline and post-NMDA; n = 6). Intrathecal co-administration of IC87201 with NMDA dose-dependently reversed the NMDA-induced reduction in tail flick latencies (thermal hyperalgesia; Figure 3B; differences among doses are significant, $P < 0.0001$, ANOVA; n = 6). NMDA-induced scratching and biting behaviour, measured in the same animals, was not affected by IC87201 (Figure 3C; $P > 0.05$, n = 6), consistent with previous findings that these behaviours could be antagonized by NMDA receptor antagonists, but not by NOS catalytic inhibitors (Fairbanks *et al.*, 2000). IC87201 inhibited motor activity (rotarod walking) in NMDA-treated mice only at 1 nmol (Figure 3D; n = 6; $P > 0.05$ for doses 1 pmol or lower; $P < 0.05$ at 1 nmol). There was no effect on motor activity at the efficacious dose of 1 pmol. When the results for thermal hyperalgesia and motor activity are normalized to percent inhibition (Figure 3E), the EC_{50} for inhibition of thermal hyperalgesia for IC87201 is around 1 pmol (95% confidence interval 0.25–4.3 pmol, n = 6; 0.05 pmol·g^{-1}, i.t.), significantly more potent than inhibition of motor coordination (though higher doses are needed to accurately determine the IC_{50} for motor coordination).

To assess the effect of IC87201 in normal nociception in naïve mice in the absence of NMDA, we measured the tail flick latencies and motor coordination after i.t. administration of IC87201. Intrathecal IC87201 exhibited apparent antinociceptive effects only at doses ≥100 pmol (5 pmol·g^{-1}; Figure 4A; n = 5). However, these are the same doses that caused ~50% inhibition of motor activity (rotarod walking) (Figure 4B; n = 5–6). Again, at the efficacious dose of 1 pmol, IC87201 had no effect on normal tail flick latency or on motor coordination (Figure 4; $P > 0.05$ between pre-dose and post-dose). Thus, the inhibition of thermal hyperalgesia by IC87201 could not be explained by inhibition of normal nociception, and there appears to be a large difference (~100–1000-fold) between the two ED50's. This contrasts with the NMDA receptor antagonist, MK801, which inhibited motor coordination (ED_{50} = 10 nmol; 95% confidence interval 2–58 nmol), normal nociception (ED_{50} = 18 nmol; 95% confidence interval 5–63 nmol) and thermal hyperalgesia; [ED_{50} = 3.2 nmol; 95% confidence interval 0.99–10 nmol (Roberts *et al.*, 2005)] with similar potencies.

Spinally administered tat-nNOS (Figure 5) also dose-dependently ($P < 0.0001$, ANOVA) inhibited the NMDA-induced reduction in tail flick latencies (Figure 5A) with an ED_{50} value of 0.3 fmol (95% confidence interval 0.1–1.0 fmol, 6–16 mice per dose, i.t.). Tat-nNOS had no effect on NMDA-induced, NOS-independent behaviours (Figure 5B; n = 6–12; $P > 0.5$). A non-transducing tat-nNOS (1–299) that is not expected to enter cells did not affect NMDA-induced thermal hyperalgesia (Figure 5A; $P > 0.5$, ANOVA; n = 6–10). In control mice, tat-nNOS also has no effect on nociception (n = 5; $P > 0.05$ for all concentrations tested, 1–1000 fmol) or rotarod performance (Figure 5C, n = 5; $P > 0.5$ at all concentrations tested). The results demonstrate that these nNOS–PSD95 disruptors are anti-hyperalgesic at concentrations that produce minimal antinociception.

IC87201 also dose-dependently inhibited NMDA-induced thermal hyperalgesia in mice after i.p. administration (Figure 6A, n = 6, $P < 0.05$) with an EC_{50} of 0.1 mg·kg^{-1} (0.3 nmol·g^{-1}; 95% confidence interval 0.01–1 mg·kg^{-1}). IC87201 had no effect on baseline tail flick latencies or motor activity in the absence of NMDA (Figure 6A,B; $P > 0.05$). Again, IC87201 minimally impaired motor coordination at the doses tested (Figure 6B; $P > 0.05$ at 0.01 mg·kg^{-1}, i.p.). We next compared the time course of this inhibition after either i.t. (1 pmol, n = 6, Figure 7A) or i.p. (1 mg·kg^{-1}, n = 4, Figure 7B) administration. Because the duration of NMDA (i.t.) action is 5 min or less, IC87201 was administered i.t. or i.p. as a pretreatment at various times prior to i.t. injection of NMDA. IC87201 was effective for 100 min after either i.t. or i.p. administration (Figure 7). The peak plasma level of 55 ng·mL^{-1} (0.2 μM, n = 3) occurred at 15 min after i.p. administration (1 mg·kg^{-1}; 3 nmol·g^{-1}) in mice. The behavioural response to i.p. administration lagged behind that of the peak plasma level by 45 min and that of i.t. administration by 30 min, probably reflecting the time needed for distribution of IC87201 to its spinal site of action following i.p. administration. Again, IC87201 did not affect tail flick baseline

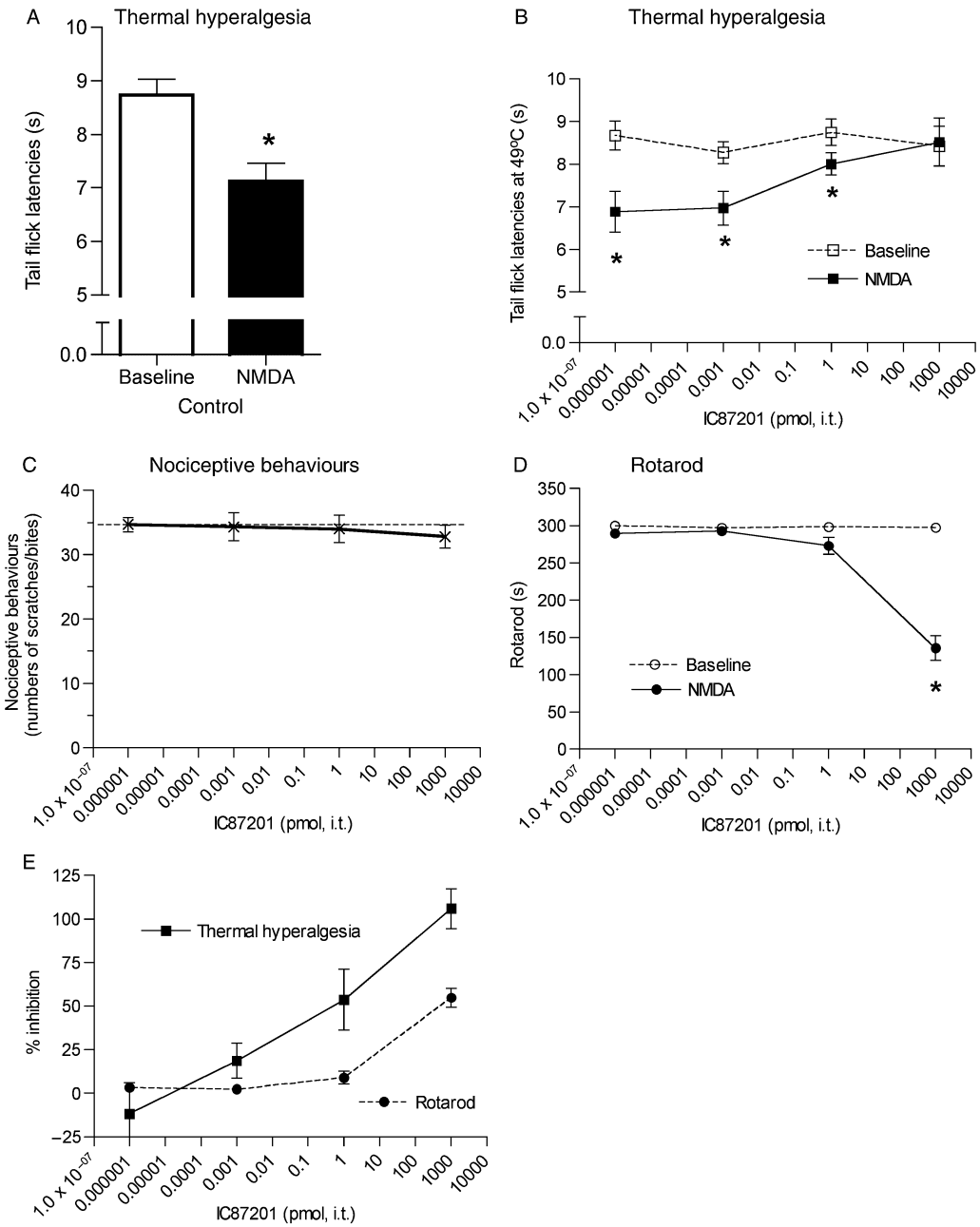

Figure 3 IC87201 is anti-hyperalgesic. (A) Intrathecal administration of NMDA-induced thermal hyperalgesia in mice, as detected by a significant reduction in warm water (49°C) tail flick latency (measured in seconds) from baseline ($P = 0.001$, as indicated by *, paired *t*-test, $n = 6$). (B) Tail flick latencies were measured in the same mice before and after NMDA and IC87201 co-administration (i.t.). IC87201 dose-dependently inhibited the NMDA-induced reduction in tail flick latencies. Baseline tail flick latencies before NMDA administration are also shown. * indicates $P < 0.05$ (using pre-dose baseline as comparator; paired *t*-test). (C) There was no effect on NMDA-evoked nociceptive behaviours measured in the same animals used in (B). (D) Motor coordination on a rotarod was also measured in the same mice before and after NMDA and IC87201 co-administration. The duration of the measurement was 300 s, so control mice stayed on the rotarod for the full 300 s. IC87201 partially impaired motor coordination only at the highest dose tested. $n = 6$ per group. (E) Results from (B) and (D) are normalized and presented as % inhibition. i.t., intrathecal; NMDA, N-methyl-D-aspartic acid.

latencies in the absence of NMDA after either i.t. (Figure 7A, $P > 0.05$) or i.p. administration (Figure 7B; $P > 0.05$).

IC87201 and tat-nNOS inhibited mechanical allodynia in the CCI model of peripheral neuropathy

Neuropathic pain symptoms develop following unilateral chronic constriction of the sciatic nerve (CCI model; Bennett and Xie, 1988). Animals with CCI show marked mechanical allodynia (measured by von Frey filaments of increasing strength) in the affected paw. In the current experiments, the baseline mean withdrawal thresholds of the injured and the uninjured paw were 8.5 g and 54.1 g respectively ($P < 0.0001$, paired *t*-test; $n = 6$).

Intrathecal administration of IC87201 dose-dependently reversed CCI-induced mechanical allodynia (Figure 8A), as

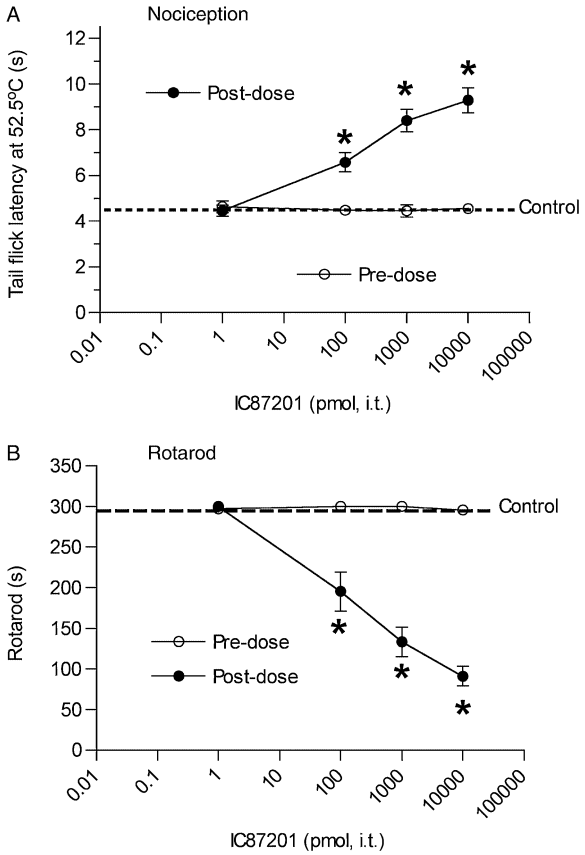

Figure 4 (A) Tail flick latencies and (B) rotarod coordination were measured (in seconds) in the same mice before (pre-dose) and after i.t. administration (post-dose) of IC87201, in the absence of NMDA. Control lines showed tail flick latency (A) or time in seconds on rotarod (B); maximum time measured was 300 s before inhibitor administration. Intrathecal IC87201 partially inhibited thermal nociception and motor coordination in naïve mice at doses ≥100 pmol. $n = 5$ per group. * indicates $P < 0.05$ (paired *t*-test using pre-dose control as comparator). i.t., intrathecal.

measured by paw withdrawal threshold. Differences among doses are significant ($P < 0.001$, $n = 6$). The onset for the 100 nmol dose (0.3 nmol·g^{-1}) was rapid – a significant effect was observed at 5 min, a full 100% inhibition was present by 30 min and measurable inhibition persisted for 2 h. During the peak effect of the small molecule, some of the drug-treated animals appeared to resume normal gait and were able to bear weight on the injured (left) paw. At 50 nmol, IC87201 ($P < 0.05$, $n = 6$) partially reversed CCI-induced mechanical allodynia while no significant effect was observed at 25 nmol ($n = 7$). No effect on mechanical thresholds of the uninjured paw was observed for the doses tested (Figure 8B; $n = 6$, $P > 0.05$). Indeed, there was no significant difference in mechanical thresholds of the uninjured paw across groups. The drug-treated animals appeared normal, with no overt signs of toxicity during the 3 h observation period. In an independent study by a contract research organization using smaller rats, i.p. administration of IC87201 (2 mg·kg^{-1}; 6.7 nmol·g^{-1}) partially reversed mechanical allodynia in the injured paw ($n = 8$, peak reversal to 71 ± 8% of pre-ligation level at 90 min). Plasma level of IC87201 peaked at 2005 ± 227 ng·mL^{-1} (6.6 μM, $n = 3$) at 15 min after i.p. administration (2 mg·kg^{-1}) in rats. Thus, IC87201 is effective in suppressing mechanical allodynia administered both i.t. and i.p.

Tat-nNOS (7 nmol or 0.02 nmol·g^{-1}, i.t.) showed a trend towards reversal of the mechanical allodynia in CCI rats (Figure 9; $n = 3$–4), though the effect was not statistically significant, when paw withdrawal threshold for each dose was compared with saline control at the corresponding time points. Tat-nNOS had no effect on withdrawal thresholds for the uninjured right paw (data not shown). Low solubility precluded testing higher concentrations of tat-nNOS.

Discussion

Neuropathic pain is a clinically challenging problem that is characterized by hyperalgesia, allodynia and spontaneous pain. The best available treatments are only moderately effective and typically limited by adverse side effects. A primary mechanism underlying the development and maintenance of neuropathic pain is NMDA-induced NO production (Ji and Strichartz, 2004). Because PSD95 is required for localization of nNOS to the NMDA receptor (Brenman *et al.*, 1996; Sattler *et al.*, 1999; Aarts and Tymianski, 2004), inhibition of nNOS binding to PSD95 would prevent functional coupling of nNOS to NMDA receptors, allowing for specific inhibition of nNOS and disruption of downstream NMDA-nNOS-mediated pain hypersensitivity. In this study, we tested two nNOS targeting inhibitors that disrupt the protein–protein interaction between PSD95 and nNOS. Both inhibitors dose-dependently attenuated NMDA-induced increases in cGMP in primary hippocampal cultures and reduced NMDA-induced thermal hyperalgesia. In addition, IC87201 abolished mechanical allodynia in the CCI model of neuropathic pain without effect on paw withdrawal for the uninjured paw. These results not only help to elucidate the relationship among PSD95, nNOS and NMDA receptors in the development of pain hypersensitivity, but also demonstrate the potential of protein–protein interaction inhibitors as novel pain therapeutics.

Disrupting the interaction between NMDA receptors and PSD95 is one approach to inhibiting the NMDA receptor–PSD95–nNOS signalling pathway. A peptide containing the protein transduction domain of the tat-protein and the last nine residues of one of the subunits of the NMDA receptor bound to PSD95 has been shown to protect neurons from focal ischaemic damage in rats (Aarts *et al.*, 2002; Sun *et al.*, 2008). As PSD95 binds to many other proteins in addition to nNOS (Sheng, 2001), disruption of the interaction between NMDA receptor and PSD95 is a less attractive therapeutic approach, as it could lead to side effects unrelated to inhibiting NOS function.

A preferable approach would be to disrupt only the interaction between PSD95 and nNOS. A GST-tagged protein containing the first 300 residues of nNOS (the same residues used in our study) was found to bind mainly the second PDZ domain of PSD95 (Cao *et al.*, 2005). When this GST-nNOS construct was transfected into cerebellar granule neurons, it inhibited glutamate-evoked activation of p38 stress-activated protein kinase and subsequent cell death (Cao *et al.*, 2005). This construct did not affect the current amplitude, time to peak current or the calcium response of the NMDA receptor,

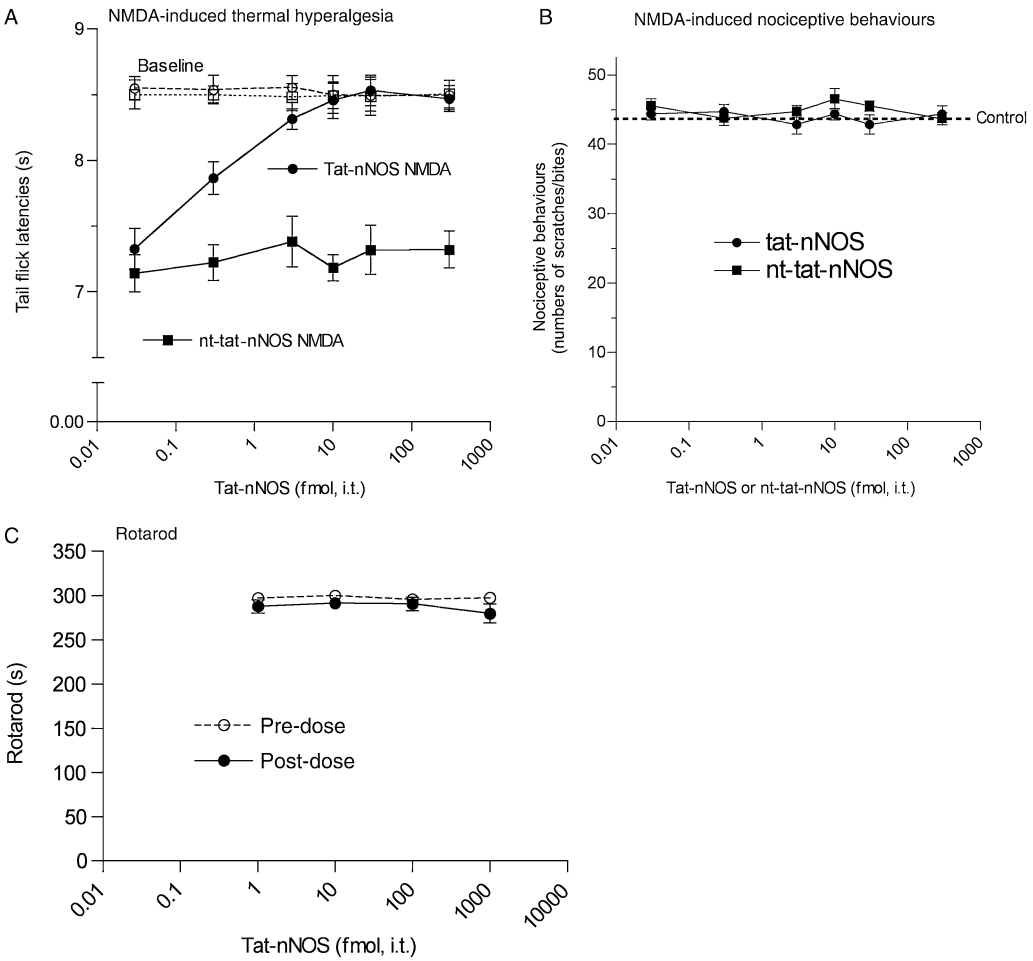

Figure 5 Tat-nNOS decreases NMDA-induced thermal hyperalgesia. (A) Tail flick latency was measured (in seconds) in the same mice before and 5 min after co-administration of NMDA and inhibitor as described in Figure 3. Tat-nNOS dose-dependently inhibited NMDA (0.3 nmol)-induced thermal hyperalgesia. A non-transducing tat-nNOS (nt-tat-nNOS) co-administered i.t. with 0.3 nmol NMDA had no effect on thermal hyperalgesia (n = 6–10). (B) Neither tat-nNOS nor nt-tat-nNOS had any effect on nNOS-independent NMDA-induced nociceptive behaviours (n = 6–12). The dotted line shows the number of scratching/biting nociceptive behaviours in NMDA-treated mice in the absence of tat-nNOS. (C) Tat-nNOS had no effect on motor coordination before or after NMDA administration. n = 5. See text session for statistical analysis. i.t., intrathecal; NMDA, N-methyl-D-aspartic acid; nNOS, neuronal nitric oxide synthase.

nor did this construct affect cell death induced by an NO donor (Cao *et al.*, 2005). While we have not tested our tat-nNOS (1–299) on NMDA receptor channel properties, it is likely that tat-nNOS (1–299) will behave similarly to GST-nNOS (1–299) and will likely have similar specificity. Both the study by Cao *et al.* and the current study confirm that inhibiting nNOS–PSD95 interactions disrupt selected aspects of NMDA receptor signalling. Moreover, the tat-fusion peptide used in the rat ischaemic damage study (Aarts *et al.*, 2002) was recently shown to be highly specific in a proteomic and biochemical analysis, though it may be more potent against the nNOS–PSD95 interaction than for the NMDA receptor–PSD95 interaction (Cui *et al.*, 2007). Injection of this peptide (dose ranges: 0.03–3 nmol·g^{-1}) administered 3 h after stroke onset significantly decreased infarct volumes measured at 24 h. Indeed, a single injection (3 nmol·g^{-1}) permanently maintained reduced infarct volumes in rats with improved neurobehaviour (including rotarod performance, motor agility, arousal/anxiety, sensorimotor, emotionality and cognitive function) compared with control (Sun *et al.*, 2008). The minimum efficacious dose of 0.3 nmol·g^{-1} (i.v.) in the rat stroke model is similar to the efficacious doses of IC87201 (0.3 nmol·g^{-1}, i.t.; 6.7 nmol·g^{-1}, i.p.) and the marginally effective dose of tat-nNOS (0.02 nmol·g^{-1}, i.t.) in a rat neuropathic pain model in this study.

While fusion proteins like GST-nNOS or tat-nNOS serve as pharmacological disruptors of protein–protein interactions, their large size and other properties make them unattractive drug candidates. IC87201 is a novel nNOS–PSD95 small molecule inhibitor that disrupted *in vitro* binding between nNOS and PSD95, and is efficacious in cell-based and *in vivo* pain models. At the minimal effective dose (MED) in the NMDA-induced thermal hyperalgesia mouse model, IC8201 had no effect on tail withdrawal latencies when administered to naïve mice, suggesting that the drug effect on tail flick latency is specifically anti-hyperalgesic and not more broadly antinociceptive. There is also close to 100-fold difference between the estimated IC_{50} values of IC87201 for inhibiting thermal hyperalgesia and for the inhibition of motor coordination, significantly better than the threefold to sixfold difference found for

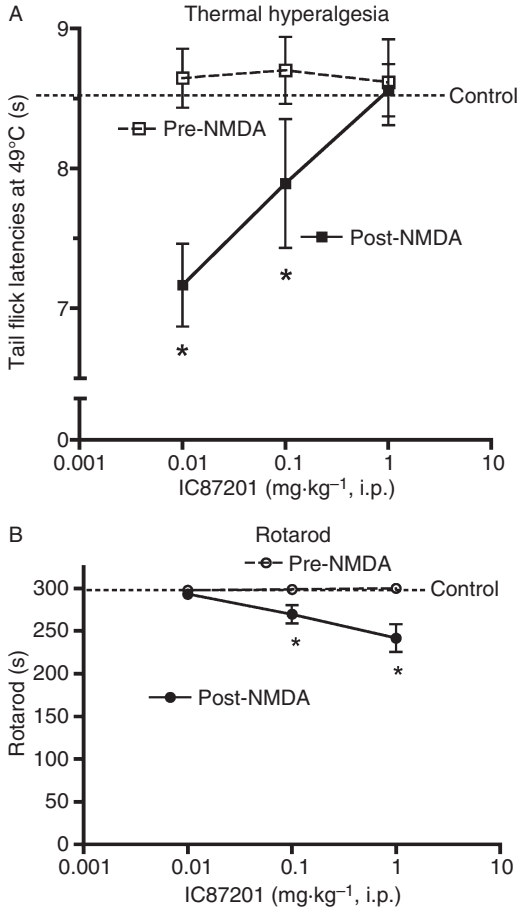

Figure 6 Systemically administered IC87201 decreases NMDA-induced thermal hyperalgesia. (A) Tail flick latencies were measured before and after i.t. administration of NMDA in the same mice. Intraperitoneal administration of IC87201 (administered 1 h before NMDA) dose-dependently attenuated NMDA-induced thermal hyperalgesia in mice, as detected by significantly reducing tail flick latency from baseline. IC87201 had no effect on baseline tail flick latency. $n = 6$. (B) The same mice were tested for motor coordination (rotarod) before and after i.t. NMDA administration. Minimal inhibition of motor activity was observed only at the highest dose tested. $n = 6$. * indicates $P < 0.05$ (paired *t*-test) for treatment group with saline as comparator. i.t., intrathecal; NMDA, N-methyl-D-aspartic acid.

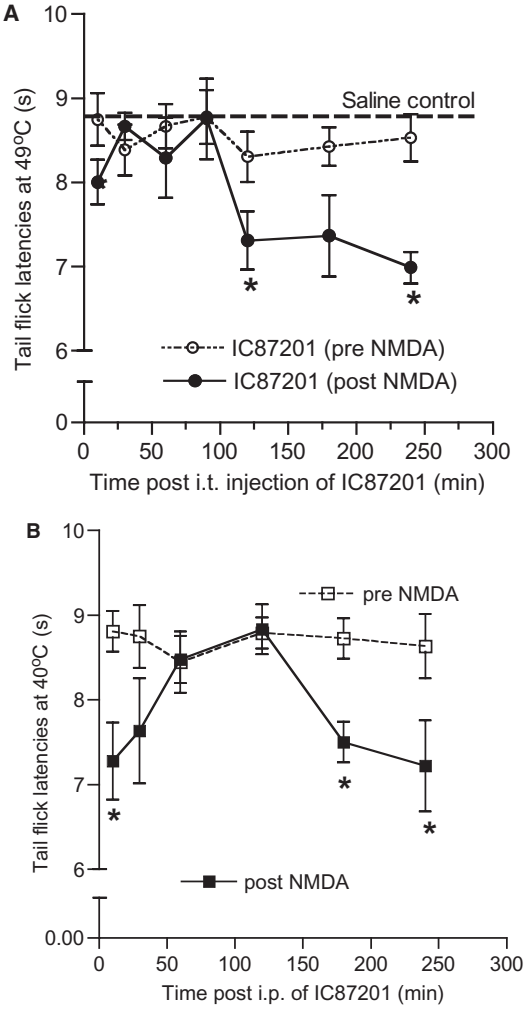

Figure 7 Intrathecal (A) and intraperitoneal (B) administration of IC87201 rapidly inhibits NMDA-induced thermal hyperalgesia. Tail flick latencies were measured before and after NMDA administration. IC87201 i.t. (1 pmol, upper panel) or i.p. (1 mg·kg^{-1}, lower panel) was administered 0–250 min prior to i.t. administration of 0.3 nmol of NMDA (i.t.). Each time point represents a separate treatment group with $n = 6$ for i.t. and $n = 4$ for i.p. administration for each IC87201 treatment group, $n = 3$ for each saline control time point. The average tail flick latency for saline controls among all the time points before NMDA administration is 8.8 ± 0.17 s and is shown as a dotted line in (A). * indicates $P < 0.05$ (paired *t*-test) for treatment groups with saline (at the corresponding time points) as comparator. i.t., intrathecal; NMDA, N-methyl-D-aspartic acid.

MK801, an NMDA receptor antagonist (Fairbanks *et al.*, 2000; Roberts *et al.*, 2005). This suggests that the reversal of NMDA-induced thermal hyperalgesia by IC87201 is unlikely to be the result of an analgesic effect. Such a selective effect has been previously observed with spinal delivery of the endogenous cannabinoid, anandamide (Richardson *et al.*, 1998) and the endogenous NMDA receptor/NOS modulator agmatine (Fairbanks *et al.*, 2000). In the rat neuropathic pain model, IC87201 fully reversed mechanical allodynia on the injured paw without affecting the mechanical threshold of the uninjured paw. The rats were able to move their injured left paws up until the withdrawal threshold was reached and resumed normal gait during the peak effect of IC87201, suggesting that the effect of IC87201 was not a result of motor impairment. Further characterization of the side effects (including motor coordination) of IC87201 and tat-nNOS in rats is needed to demonstrate whether these inhibitors offer improved therapeutic index over existing therapies in this or similar disease models.

The *in vivo* potency of IC87201 in the mouse NMDA-induced thermal hyperalgesia model was 1 pmol (0.05 pmol·g^{-1}) for i.t. administration, or 0.2 μM at the injection site, which is much more potent than the estimated cell-based EC_{50} of 2.7 μM or the *in vitro* IC_{50} of 31 μM. While we were not able to measure spinal level of IC87201 after i.t. administration, the plasma level of IC87201 did reach close to μM range after i.p. administration in mice and rats. Anandamide was also more potent in a carrageenan-induced thermal hyperalgesia mouse model with the MED of 0.07 fmol (or 14 fM, 0.0028 fmol·g^{-1}; i.t.), while its K_d for receptor binding was in the nM range, and MED for inhibition of potassium or capsaicin-induced release of CGRP from rat spinal cord

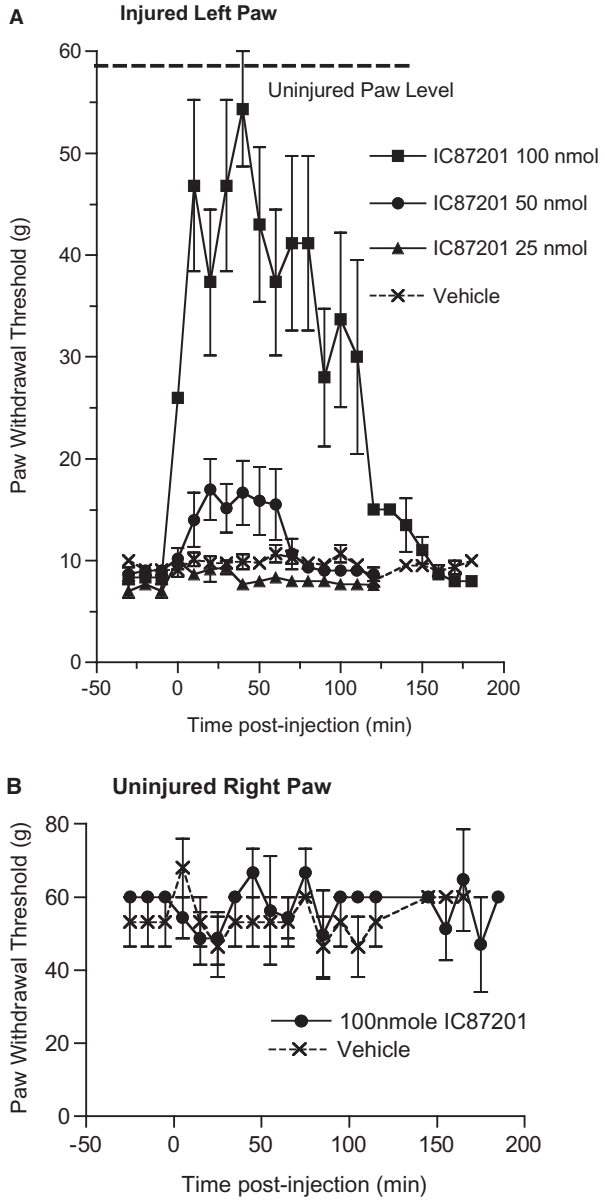

Figure 8 IC87201 decreases mechanical allodynia in a rat model of neuropathic pain. Intrathecal administration of IC87201 (100 nmol per 10 μL infusion via spinal catheter; $n = 6$) completely reversed mechanical allodynia in the injured left paw (A) of rats with CCI. At 50 nmol ($n = 6$), IC87201 had partial efficacy, while no significant effect was observed for the 25 nmol dose ($n = 7$). IC87201 (100 nmol) and vehicle had no effect on withdrawal thresholds for the uninjured right paw (B). Mechanical allodynia (as assessed by calibrated von Frey filaments) developed between 1 day and 7 days after the constriction injury. IC87201 ($n = 6$) or vehicle ($n = 3$–5) was administered i.t., and behavioural testing continued at 5 min intervals for at least 2 h. Statistical analysis for treatment group was repeated measures ANOVA. Time points for IC87201 (100 nmol) at 10, 30, 40, 50, 70 and 80 min were significantly different ($P < 0.05$) compared with pretreatment group and to vehicle treatment group at the corresponding time points. ANOVA, analysis of variance; CCI, chronic constriction injury.

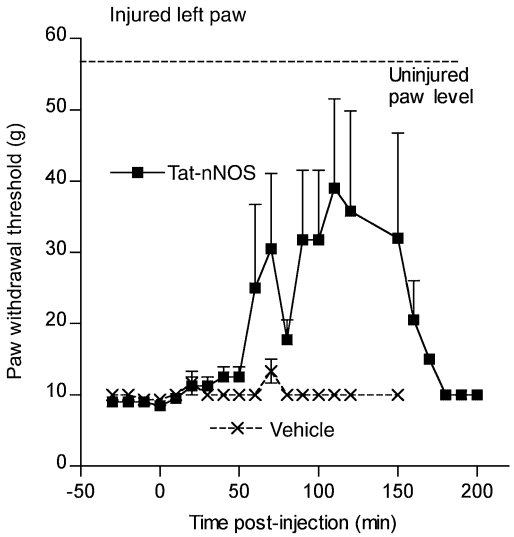

Figure 9 Effect of tat-nNOS on mechanical allodynia in CCI rats. Tat-nNOS (7 nmol, i.t., $n = 3$–4 from −30 min to 120 min, $n = 2$ for 150–200 min) partially reversed mechanical allodynia in the injured left paw of CCI rats. Vehicle alone had no effect. i.t., intrathecal; CCI, chronic constriction injury; nNOS, neuronal nitric oxide synthase.

cultures were 100 nM and 100 pM respectively (Richardson *et al*., 1998). Similar to that study, IC87201 and tat-nNOS may show better efficacy in hypersensitized animals because of synergism with other ligands and proteins or change in the affinities of the receptors or proteins involved. Even in 'normal' state, the presence of other proteins in the physiological NMDA receptor–PSD95–nNOS complex may change the affinities of the individual pairs of protein partners. Furthermore, it is possible that disrupting only a small fraction of nNOS and PSD95 interactions will result in a large inhibition of downstream signalling pathways. The higher potency of IC87201 after i.t. administration compared with systemic administration as well as a delay in the onset of action after i.p. administration may suggest a spinal site of action.

It is impossible to rule out possible effects of IC87201 and tat-nNOS at other targets. While the two inhibitors have a similar IC_{50} in the NMDA-induced cGMP cell assay, tat-nNOS is more potent in the mouse hyperalgesia model. However, this cell-based assay has not been demonstrated to be predictive of *in vivo* potency and other factors such as protein binding of the small molecule, and differences in tissue sequestration may explain some of the differences between *in vitro* and *in vivo* potencies. Furthermore, given their very different molecular structures, it seems unlikely that a small molecule and a tat-fusion protein would inhibit pain hypersensitivity in mice and rats through the same putative 'off-target' mechanism, resulting in efficacy as anti-hyperalgesics. Preliminary studies suggest that IC87201, at 10 μM, had no significant effect on binding on a panel of 34 targets that included receptors, transporters and ion channels (data not shown). Further characterization, beyond the scope of the current study, of IC87201 will be needed to reveal additional site(s) of action that may explain its anti-hyperalgesic effects. These caveats notwithstanding, these targeting disruptors appear to offer the advantage of selectively inhibiting the NMDA–PSD95–nNOS arm of the NMDA receptor signalling pathway, downstream of NMDA receptor activation.

Both tat-nNOS and IC87201 show promises for treating neuropathic pain while minimizing some of the side effects

typically associated with NMDA receptor antagonists or non-selective NOS catalytic inhibitors (Vallance and Leiper, 2002). These targeting inhibitors join a small but growing family of protein–protein interaction disruptors with demonstrated efficacy in *in vivo* disease models. These inhibitors, similar to the tat-fusion proteins used by Aarts *et al*. (2002), are also likely to be efficacious in treating ischaemic brain injury as well as other neurological diseases where detrimental NMDA receptor signalling plays a major role. More importantly, these results validate the concept that protein targeting could be a practical and novel approach to selectively disrupt cellular function while avoiding adverse effects associated with more general inhibition of receptor or enzyme function.

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Conflict of interest

The authors state no conflict of interest.

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