Current Biology, Vol. 13, 321–328, February 18, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00084-8

Neuropathic Sensitization of Behavioral Reflexes and Spinal NMDA Receptor/CaM Kinase II Interactions Are Disrupted in PSD-95 Mutant Mice

Emer M. Garry,[1] Andrew Moss,[1,4] Ada Delaney,[1] Francis O'Neill,[1] James Blakemore,[1,5] Julian Bowen,[1] Holger Husi,[2] Rory Mitchell,[3] Seth G.N. Grant,[2] and Susan M. Fleetwood-Walker[1,*]

[1]Division of Preclinical Veterinary Sciences, R(D)SVS
University of Edinburgh
Summerhall, Edinburgh EH9 1QH
United Kingdom

[2]Division of Neuroscience
University of Edinburgh
1 George Square
Edinburgh EH8 9JZ
United Kingdom

[3]MRC Membrane and Adapter Proteins Co-operative Group and Membrane Biology Group
Division of Biomedical and Clinical Laboratory Sciences
University of Edinburgh
Edinburgh EH8 9XD
United Kingdom

Summary

Chronic pain due to nerve injury is resistant to current analgesics. Animal models of neuropathic pain show neuronal plasticity and behavioral reflex sensitization in the spinal cord that depend on the NMDA receptor [1, 2]. We reveal complexes of NMDA receptors with the multivalent adaptor protein PSD-95 [3, 4] in the dorsal horn of spinal cord and show that PSD-95 plays a key role in neuropathic reflex sensitization. Using mutant mice expressing a truncated form of the PSD-95 molecule [5], we show their failure to develop the NMDA receptor-dependent hyperalgesia and allodynia seen in the CCI model of neuropathic pain [6], but normal inflammatory nociceptive behavior following the injection of formalin. In wild-type mice following CCI, CaM kinase II inhibitors attenuate sensitization of behavioral reflexes, elevated constitutive (autophosphorylated) activity of CaM kinase II is detected in spinal cord, and increased amounts of phospho-Thr^{286} CaM kinase II coimmunoprecipitate with NMDA receptor NR2A/B subunits. Each of these changes is prevented in PSD-95 mutant mice although CaM kinase II is present and can be activated. Disruption of CaM kinase II docking to the NMDA receptor and activation may be responsible for the lack of neuropathic behavioral reflex sensitization in PSD-95 mutant mice.

*Correspondence: s.m.fleetwood-walker@ed.ac.uk

[4]Present address: Department of Anatomy and Developmental Biology, Medawar Building, University College London, Gower St., London WC1E 6BT, United Kingdom.

[5]Present address: Cell Factors plc, St. John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, United Kingdom.

Results

PSD-95 Expression in Spinal Cord

Using histochemical staining for the β-galactosidase reporter gene, we showed that PSD-95 expression is specifically restricted to lamina II of the spinal dorsal horn, a prime location for the processing of nociceptive afferent inputs (Figure 1A), and is found throughout lumbar and thoracic spinal cord (Figure 1B). This distribution overlaps with those of NMDA receptor subunits (particularly NR2B) throughout lumbar and thoracic spinal cord [2]. PSD-95 expression was not detected in dorsal root entry zones (Figure 1B, arrow), dorsal root ganglion (DRG), dorsal roots, or sciatic nerve (data not shown). Using coimmunoprecipitation, we could isolate NMDA receptor complexes from spinal cord that contained NR1, NR2A, NR2B, and PSD-95, similar to those found in forebrain [3, 4] (Figure 1C).

PSD-95 Mutant Mice Lack Behavioral Reflex Sensitization Following CCI

We used the CCI model of neuropathic pain to examine the effects of sciatic nerve injury in homozygous PSD-95 mutant mice and their wild-type littermates. Over 7–10 days following CCI (under halothane anesthesia), wild-type mice progressively developed marked ipsilateral thermal hyperalgesia (reduced paw withdrawal latency, PWL; Figure 2A), mechanical allodynia (reduced paw withdrawal threshold, PWT; Figure 2C), and cold allodynia (increased suspended paw elevation time, SPET; Figure 2E). All responses from the contralateral hind limb remained unaltered. Thermal hyperalgesia and mechanical allodynia were absent in PSD-95 mutant mice (Figures 2B and 2D), while cold allodynia was severely attenuated, reaching statistical significance at only one time point (Figure 2F). No differences in motor function or coordination (as measured by the rotarod test) were observed in PSD-95 mutants compared to wild-type mice. In addition, there was no evidence for anatomical alterations in afferents of the mutant mice, with similar axon diameter and myelin thickness profiles for Aβ, Aδ, and C fibers between naive wild-type and homozygous PSD-95 mutant mice (in accordance with previous findings [7, 8]), and the extent of CCI-induced changes in afferent fibers was unaltered.

PSD-95 Mutant Mice Display Normal Responses to Inflammatory Nociceptive Stimuli

Sensitization of nociceptive reflexes can also be brought about by peripheral inflammatory stimuli [9], but inflammatory and neuropathic states display a number of distinct characteristics [1, 10]. We therefore tested whether PSD-95 mutant mice also display a deficit in behavioral responses to inflammation by intraplantar injection of formalin (10 μl of 1.5% solution in saline, during brief halothane anesthesia [11, 12]). PSD-95 mutant mice and wild-type littermates displayed the same responses of paw licking and flicking in the early acute phase of the

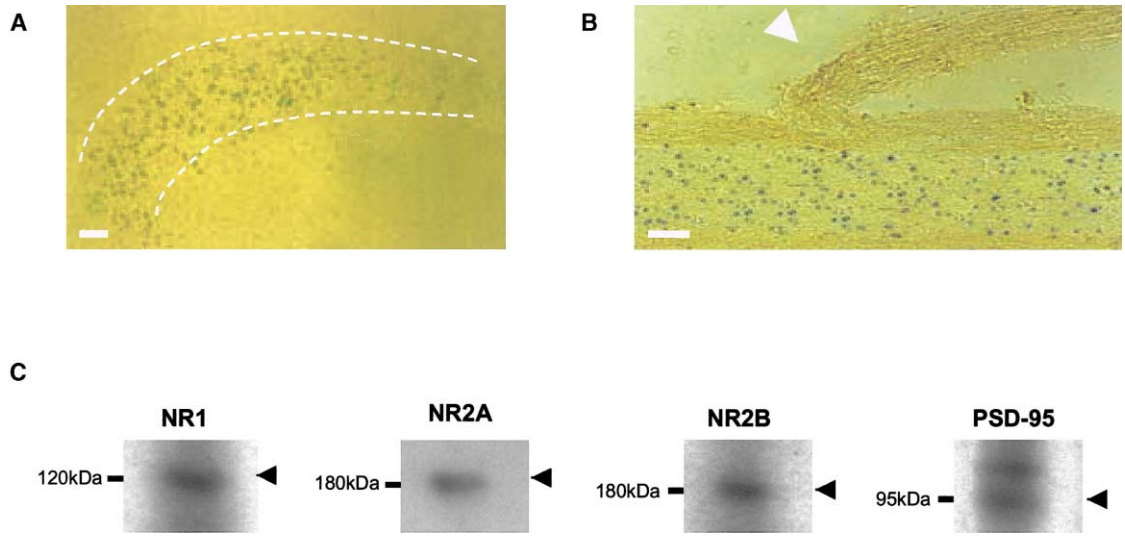


Figure 1. Expression of PSD-95 and Components of the NMDA Receptor Complex in Spinal Cord

Heterozygous PSD-95 (wt/mutant) animals (n = 5) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. 10 μm sections were washed with ice-cold PBS/$MgCl_2$ and then 0.041% $MgCl_2$, 0.01% Na deoxycholate, 0.02% Nonidet-NP40, in 0.1 M PBS. Sections were stained at 22°C in 0.042% potassium ferrocyanide, 0.033% potassium ferricyanide in detergent buffer with 1 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and incubated at 37°C in the dark for 4–6 hr.

(A) Transverse section, showing specific distribution of β-galactosidase-positive cells in lamina II (outlined with dashed line) of the superficial dorsal horn.

(B) Longitudinal section showing staining in dorsal horn, but not at the site of dorsal root entry (arrow). Scale bar equals 10 μm.

(C) Immunoblots for the presence of NMDA receptor complex proteins in NR2A/B-directed immunoprecipitates of L3-L6 spinal cord from wild-type mice.

formalin test (5–10 min following injection), and the late (inflammatory) phase of the response (25 min to 1 hr) was also indistinguishable between the mutant and wild-type mice (Figure 2G), in striking contrast to the lack of neuropathic behavioral reflex sensitization seen in the mutants. The degree of peripheral inflammation as assessed by paw volume was the same for PSD-95 mutant and wild-type mice.

NMDA Receptor-Dependent Hyperalgesia and Allodynia in Wild-Type, but not PSD-95 Mutant Mice

Intrathecal administration of the selective NMDA receptor antagonist (R)-CPP (under brief halothane anesthesia) completely reversed thermal hyperalgesia and mechanical allodynia that had developed ipsilateral to CCI in wild-type mice, with no detectable effect on contralateral responses or in naive animals. At 10–30 min following injection of (R)-CPP (100 pmole), the ipsilateral: contralateral differences in PWL and PWT (thermal hyperalgesia and mechanical allodynia) were reduced to 11.6% ± 5.9% and 8.3% ± 3.5% of predrug differences ($n = 6$, $p < 0.05$ by Wilcoxon test in each case). Recovery was complete by 60–80 min. The equivalent values for saline were 94.8% ± 3.9% and 90.0% ± 5.3%, respectively. The behavioral changes brought about following CCI were bilaterally mimicked in naive wild-type mice by intrathecal administration of NMDA (250 pmole), showing a 45.9% ± 5.1% reduction in mechanical paw withdrawal threshold (PWT) and a 36.2% ± 3.9% reduction in thermal paw withdrawal latency (PWL) over 10–30 min following injection ($p < 0.05$ in each case by Mann-Whitney U test). In contrast, behavioral responses of PSD-95 mutant mice following CCI were unaffected by intrathecal administration of (R)-CPP, with PWL and PWT values (which, as described, displayed no sensitization in these animals) remaining at 98.4% ± 7.2% and 94.5% ± 4.9% of predrug controls. Correspondingly, naive PSD-95 mutants showed no changes in PWL and PWT following intrathecal injection of NMDA, with values remaining at 96.4% ± 5.0% and 99.4% ± 6.3% of predrug controls. These findings suggest that NMDA receptor:PSD-95 interactions in spinal cord play a key role in the development of neuropathic behavioral reflex sensitization. In both wild-type mice and PSD-95 mutant mice, the second (inflammatory) phase of the nociceptive reflex response to formalin was completely inhibited by intrathecal injection of 100 pmol (R)-CPP. The mean number of paw flinches/flicks over the peak 15 min of the second phase formalin response was reduced by (R)-CPP to 11.5% ± 4.3% of control in wild-type and by 10.1% ± 5.2% in PSD-95 mutants ($n = 4$; $p < 0.05$ by Student's t test). Intrathecal injection of saline had no detectable effect on formalin responses with corresponding values remaining within 6% of those from uninjured controls. This indicates that while this inflammatory nociceptive behavioral response involves the NMDA receptor, it does not share the additional requirement for intact PSD-95 that is seen in the behavioral reflex sensitization induced by CCI.

CaM Kinase II Inhibitors Alleviate Neuropathic Sensitization

NMDA receptor:PSD-95 complexes in the forebrain incorporate the Ca^{2+}-dependent protein kinase, CaM kinase II, docked to NR2 subunits [3, 4, 13–15], where it

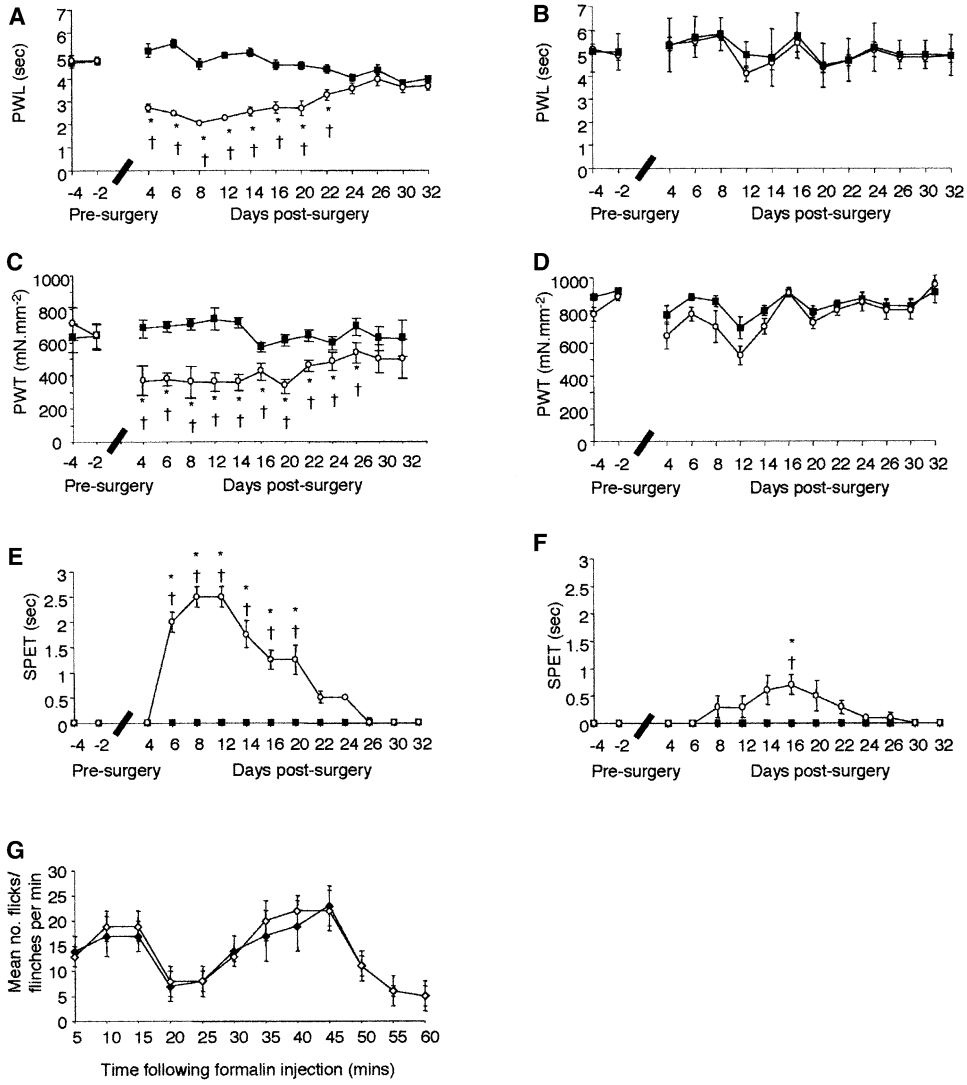


Figure 2. Behavioral Analysis of Wild-Type and PSD-95 Mutant Mice with Chronic Constriction Injury (CCI) to the Sciatic Nerve

Data show mean ± SEM responses for each day before and following the induction of CCI. A variation of the chronic constriction injury (CCI) model for rat [6] was used, whereby (under halothane anesthesia) three ligatures separated by 1 mm were tied loosely to constrict the tibial branch of the sciatic nerve.

(A and B) In wild-type mice (A), paw withdrawal latency (PWL) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to CCI (open circle) showed significant differences between postoperative and preoperative values (dagger indicates $p < 0.05$; Kruskal-Wallis one-way ANOVA) and from postoperative, contralateral (closed square) values (asterisk indicates $p < 0.05$ by Student's t test). No thermal hyperalgesia was seen on the contralateral side (closed square) of wild-type mice (A) or in PSD-95 mutant mice (B) on either side.

(C and D) Paw withdrawal thresholds (PWT) from mechanical stimulation (von Frey filaments) for wild-type mice showed significant differences between postoperative and preoperative values on the side ipsilateral (open circle) to CCI (dagger indicates $p < 0.05$; Dunn's Method ANOVA on ranks) and between postoperative ipsilateral and contralateral values (asterisk indicates $p < 0.05$, Mann-Whitney U test). No significant differences were seen on the contralateral side (closed square) of wild-type mice (C) following nerve ligation or in PSD-95 mutants (D).

(E and F) The suspended paw elevation time (SPET) in response to a cold water (4°C) stimulus is shown for the ipsilateral paw (open circle) and contralateral paw (closed square) in wild-type mice (E) and in PSD-95 mutant mice (F). Following nerve ligation, SPET scores for the contralateral paw were always zero for both wild-type and mutant mice. Statistically significant differences between postsurgery and presurgery ipsilateral values are indicated by a dagger ($p < 0.05$, Dunn's Method ANOVA on ranks), and an asterisk ($p < 0.05$, Mann-Whitney U test) indicates statistically significant differences between postsurgery ipsilateral and contralateral responses.

(G) Data represent the number of paw flinch/flick responses per minute following the intraplantar injection of formalin. There was no significant difference between responses of wild-type (closed diamond) and PSD-95 mutant (open diamond) mice in either the initial stage or the second (inflammatory) stage of this test. $n = 5–9$ in each case.

is predicted to play a particular role in NMDA receptor-mediated synaptic plasticity [16]. Recent studies indicate that Ca^{2+}/calmodulin stimulation induces autophosphorylation at Thr^{286} CaM kinase II and docking of the active kinase to the C-terminal domain of NR2B [17]. We first investigated whether CaM kinase II is necessary for the NMDA receptor-dependent sensitization of spinal neurons in CCI. Intrathecal administration of the se-

Table 1. Effects of Acute Topical Drug Administration and of CCI on CaM Kinase II Activation in Spinal Cord of Wild-Type and PSD-95 Mutant Mice

Topical Application to Spinal Dorsal Horn	Constitutive CaM Kinase II Activity (% of Maximal Activity Evoked In Vitro by Ca^{2+}/Calmodulin)			
	Wild-Type		PSD-95 mutant	
In Naive Mice				
Saline	16.7 ± 3.1		13.9 ± 2.7	
NMDA/glycine (150 nmole/30 nmole)	39.4 ± 3.4*		37.0 ± 4.1*	
Ionomycin (5 nmole)	43.9 ± 2.7*		44.8 ± 5.2*	
In Mice with Established CCI	CCI Ipsi	CCI Contra	CCI Ipsi	CCI Contra
Saline	28.8 ± 3.1*	16.8 ± 2.0	16.1 ± 2.1	14.9 ± 1.7
(R)-CPP (5 nmole)	14.3 ± 1.5	17.5 ± 3.0	-	-

Agents were topically applied in a volume of 500 μl for 15 min to the dorsal surface of L3-L6 spinal cord before rapid removal of tissue and homogenization prior to CaM kinase II immunoprecipitation and kinase activity assay. The concentrations of NMDA/glycine and ionomycin used were selected to provide maximal intensity stimuli and ensure detectability of changes in enzyme activity within the heterogeneous tissue samples taken. The mean maximal CaM kinase II activity in immunoprecipitates from naive PSD-95 mutant mice spinal cord (stimulated by Ca^{2+}/calmodulin addition in vitro) was unaltered from that in wild-type mice (108.3 ± 13.7 and 97.8 ± 13.1 pmoles ^{33}P/min/μg original extract protein, respectively). Data are expressed as the percentage of maximal CaM kinase II activity (means ± SEM, n = 8–10). Statistically significant differences are indicated by asterisk ($p < 0.05$ by Mann Whitney U test, compared to corresponding saline values in naive mice and by Wilcoxon test compared to corresponding contralateral values in mice with established CCI).

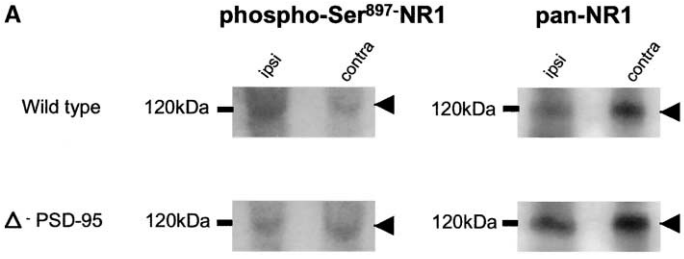
lective CaM kinase II inhibitors, KN-93 (120 pmole [18]) and myristoyl-autocamtide 2-related inhibitory peptide (myr-AIP, myr-KKALRRQEAVDAL, 1 nmole [19]), clearly reversed the neuropathic thermal hyperalgesia and mechanical allodynia seen in wild-type mice following CCI, whereas a control myristoylated peptide (myr-GRRNAIHDE, 1 nmole) or saline vehicle were without effect. Cell permeability of myr-AIP has been documented [19], and we have shown that it can effectively attenuate CaM kinase II autophosphorylation in spinal cord following topical application (unpublished results). At 10–30 min following injection, the ipsilateral:contralateral difference in PWL (thermal hyperalgesia) was significantly reduced to 18.2% ± 3.6%* and 21.2 ± 2.3%* of predrug control values by KN-93 and myr-AIP, respectively (n = 6–9, *$p < 0.05$ by Wilcoxon test), but not by myr-control peptide (90.5% ± 8.6%) or saline (above). Corresponding values for the lateral difference in PWT (mechanical allodynia) were 3.3% ± 1.4%, 37.3% ± 5.9%*, and 96.3% ± 5.8%. Recovery from the effects of each reagent was largely complete by 60–80 min. Thus, the NMDA receptor-dependent sensitization of spinal neurons that is lacking in PSD-95 mutant mice crucially requires CaM kinase II to exert its functional influence.

CaM Kinase II Activation in Response to Spinal NMDA Receptor Stimulation or CCI

We directly monitored the activation state of spinal cord CaM kinase II, isolated by immunoprecipitation from tissue extracts. Following CCI, the proportion of CaM kinase II activity that was constitutive (a read-out of previous activation by Ca^{2+}/calmodulin) was significantly greater in spinal cord ipsilateral to nerve injury compared to contralateral in wild-type animals, but not in PSD-95 mutants (Table 1). This CCI-induced increment could be completely prevented by topical (R)-CPP, confirming the requirement for NMDA receptors in mediating the necessary Ca^{2+} entry. The maximal activity of CaM kinase II from spinal cord that could be evoked in vitro by Ca^{2+}/calmodulin addition was unchanged between wild-type and PSD-95 mutant mice. Furthermore, topical application of a maximally effective concentration of NMDA (with glycine) or of ionomycin elicited the same extent of CaM kinase II activation in naive PSD-95 mutant mice as seen in wild-type littermates (Table 1). Thus, the spinal complement of CaM kinase II is fully able to respond to Ca^{2+}-elevating stimuli in PSD-95 mutant mice. Nevertheless, the NMDA receptor-mediated Ca^{2+} entry that occurs physiologically during CCI appears to activate CaM kinase II more effectively in wild-type than in PSD-95 mutant mice. PSD-95 therefore seems to play an important role in facilitating the functional coupling between the NMDA receptor and CaM kinase II in CCI.

Molecular Mechanisms Disrupted in PSD-95 Mutant Mice

Changes either in protein phosphorylation or in protein:protein interactions might underlie the facilitated NMDA receptor:CaM kinase II coupling in CCI. The NMDA receptor is known to be phosphorylated by PKC and PKA at Ser^{896} and Ser^{897}, respectively, within the NR1 subunit [20], and PSD-95 is thought to bind the PKA/PKC-docking protein AKAP79/150 [21] through domains that are lacking in the mutant PSD-95 protein here, suggesting that regulatory phosphorylation of the NMDA receptor may be disturbed in the mutant mice. Figure 3A shows that NR1-directed immunoprecipitates demonstrated a small increase in phospho-Ser^{897}-NR1 immunoreactivity ipsilateral to CCI, which tended to be less in PSD-95 mutant mice. No clear signal was obtained for phospho-Ser^{896} immunoreactivity. Pan-NR1 control blots were also carried out to confirm that the proportion of NR1 phosphorylated at Ser^{897} was increased (Figure 3A). Small reductions in the levels of pan-NR1 immunoreactivity were seen ipsilateral to CCI (in accordance with a previous report [22] and our unpublished data from the rat CCI model), and these reductions were similar in PSD-95 mutant mice to those in

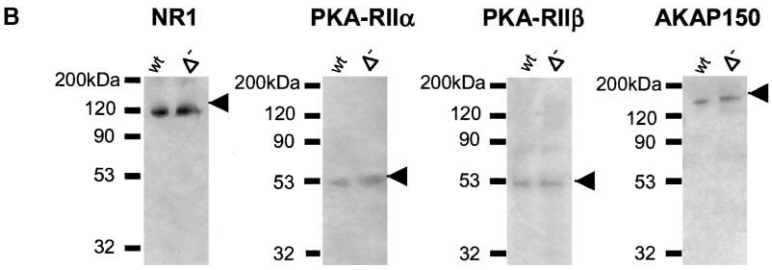


Mean relative phospho-Ser^{897}NR1: pan NR1 immunoreactivity (grey scale ratios relative to wt contra)	
Wild type ipsi	4.67 ± .63
Wild type contra	1.0 (as defined)
Δ$^-$ ipsi	1.35 ± 0.22
Δ$^-$ contra	1.02 ± 0.17

Figure 3. Immunoblots for Phospho-Ser^{897}-NR1 and PKA-Related Proteins in NR1 and NR2 Immunoprecipitates from Wild-Type and PSD-95 Mutant Mice Either Following CCI Surgery or in Naive Samples

(A) Western blot analysis of NR1 immunoprecipitates from spinal cord following sciatic CCI carried out 12 days previously. Immunoprecipitates from wild-type (wt) or PSD-95 mutant (open triangle) mice, ipsilateral or contralateral to the injury, were probed for phospho-Ser^{897}-NR1 or pan-NR1 (n = 5). The mean ratio of phospho-Ser^{897}-NR1:pan-NR1 immunoreactivity appeared to be increased in wild-type ipsilateral samples, but not those from the mutant mice. Approximate molecular weights are shown on the left.

(B) Western blot analysis of spinal cord proteins captured by "pep6" NR2B C-terminal (SIESDV) peptide-affinity resin [4]. Samples derived from wild-type (wt) or PSD-95 mutant (open triangle) mice were probed with antibodies for NR1, PKA-RIIα, PKA-RIIβ, and AKAP150 (n = 8). There was no detectable difference in the levels of any of these captured proteins when wild-type extracts were compared to PSD-95 mutant extracts.



wild-type littermates. However, affinity capture of NMDA receptor complexes and associated proteins using an NR2B tail peptide affinity resin [3, 4] showed consistently no changes in NR1, PKA-RIIα, PKA-RIIβ, or AKAP150 immunoreactivity between complexes from wild-type or PSD-95 mutant spinal cord (Figure 3B). This suggests that altered AKAP-mediated kinase targeting to the NMDA receptor complex in the PSD-95 mutant mice does not underlie their neuropathic sensitization-resistant phenotype.

To examine the coexistence of CaM kinase II and NMDA receptors in molecular complexes in the spinal cord following CCI and to assess any difference caused by the mutant PSD-95 protein, we immunoprecipitated NR2A/B subunits from spinal cord following CCI and probed for the levels of total and autophosphorylated (activated) CaM kinase IIα immunoreactivity bound to the receptor. In wild-type spinal cord, we found high levels of CaM kinase IIα associated with the receptor, with an increase in CaM kinase IIα levels ipsilateral to CCI compared to contralateral (Figure 4A). Similarly, there was a marked increase in the levels of NR2A/B bound phospho-Thr^{286} CaM kinase IIα ipsilateral to injury in wild-type mice (Figure 4B) where under the same conditions in PSD-95 mutant mice, minimal levels of CaM kinase IIα (Figure 4A) or phospho-Thr^{286} CaM kinase IIα (Figure 4B) were bound to NR2A/B subunits. When blots were probed for control pull-down of NR2A and NR2B subunits, uniform immunoreactivity was seen in wild-type and PSD-95 mutant tissue (Figure 4C). Similarly, the tissue levels of CaM kinase II expression were unaltered between wild-type and PSD-95 mutant mice (Figure 4D). These data reveal a profound loss of NMDA receptor-associated CaM kinase IIα and phospho-Thr^{286} CaM kinase IIα (an autonomously active form of the enzyme) in the PSD-95 mutant mice. Disruption of this interaction could provide a unified explanation of the linked dependence of sensitization on NMDA receptors and CaM kinase II, together with the lack of CCI-induced behavioral reflex sensitization and CCI-induced CaM kinase II activation seen in the PSD-95 mutants.

Discussion

Using transgenic mice and a behavioral reflex model of central sensitization following nerve injury [5, 6], we have

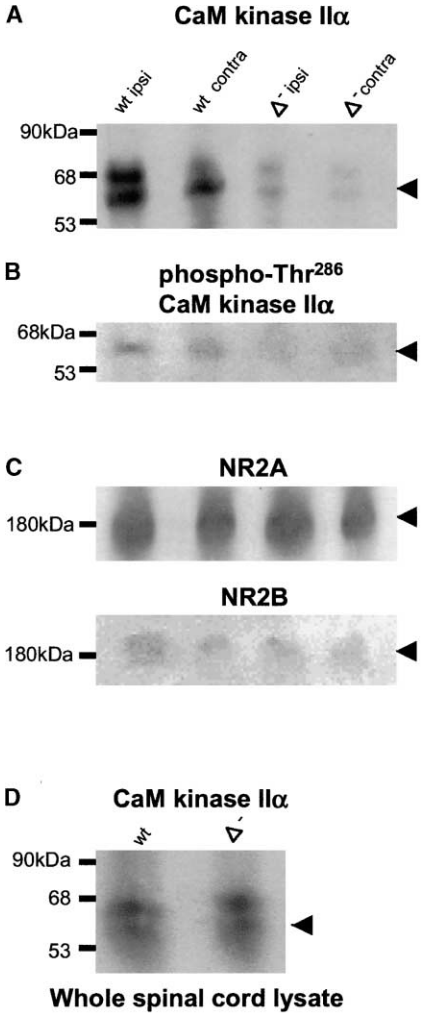


Figure 4. Immunoblots for CaM Kinase IIα Association with Spinal NR2A/B Immunoprecipitates

(A) Western blot of NR2A/B immunoprecipitates from wild-type (wt) and PSD-95 mutant (open triangle) spinal cord at the peak of behavioral sensitization following CCI (n = 8). Ipsilateral to injury (ipsi) wild-type mice show increased levels of receptor bound CaM kinase II when compared to the contralateral (contra) spinal cord. In comparison, the levels of receptor bound CaM kinase II in PSD-95 mutant spinal cord were greatly reduced both ipsilateral and contralateral to injury. Approximate molecular weights are shown on the left.

(B) Western blots probed for phospho-Thr^{286} CaM kinase II show increased levels of NR-associated immunoreactivity ipsilateral to nerve injury in wild-type mice, but not PSD-95 (open triangle) mutants.

(C) Recovery of NR2A and NR2B from the immunoprecipitates showed no detectable differences between wild-type and PSD-95 mutant samples.

(D) Western blot of whole spinal cord lysate from naive wild-type (wt) mice and PSD-95 mutant mice (open triangle) probed for CaM kinase II shows that the total content of CaM kinase II was similar in wild-type and PSD-95 mutant mice.

addressed the role of the NMDA receptor-adaptor protein PSD-95 in neuropathic pain. Histochemical staining for the β-galactosidase reporter incorporated into the mutant construct showed specific expression in many cells within lamina II of the superficial dorsal horn. This corresponds to regions of high NMDA receptor expression, to the termination of fine sensory afferents, and matches a recent report of native PSD-95 immunoreactivity in spinal cord [23]. In spinal cord of wild-type mice, NR2 subunit immunoprecipitates additionally pulled down both NR1 and PSD-95, confirming the presence of spinal complexes of NMDA receptor subunits with PSD-95.

The functional impact of the mutant PSD-95 construct on neuropathic behavioral reflex sensitization was striking. Thermal hyperalgesia, mechanical allodynia, and cold allodynia brought about by peripheral nerve injury were all virtually absent in homozygous PSD-95 mutant mice, despite the entirely undiminished nociceptive reflex behavior that they displayed following intraplantar formalin. The enduring lack of neuropathic sensitization seen in this phenotype is consistent with a brief report of transient delays in the development of such sensitization following a PSD-95 antisense reagent [23]. The current report reveals for the first time that the requirement for PSD-95 is both selective for neuropathic rather than inflammatory sensitization and is absolute. Intrathecal injection of NMDA receptor antagonist confirmed that the neuropathic hyperalgesia and allodynia are dependent on spinal NMDA receptors, matching other results in neuropathic pain models [1]. Similar sensitivity to NMDA receptor antagonist was seen for the late phase inflammatory formalin response, as in other reports [11, 12]. Intrathecal injection of NMDA did not produce hyperalgesia and allodynia in PSD-95 mutant mice, at a dose that was effective in naive wild-type mice. This is consistent with a recent report of attenuated NMDA-induced thermal hyperalgesia in the tail flick test when animals were treated with a PSD-95 antisense reagent [24]. The present data demonstrate that failure of a key signal from spinal NMDA receptors in the PSD-95 mutant mice is likely to underlie their inability to develop neuropathic sensitization. Inflammatory sensitization, although also requiring NMDA receptors, appears to be independent of such PSD-95-mediated coupling of the NMDA receptor to other proteins.

The sensitivity of NMDA receptor/PSD-95-dependent neuropathic sensitization to CaM kinase II inhibitors suggests that this enzyme may play a key role downstream of the complex in producing the sensitized state. This idea is consistent with evidence that NMDA receptor-mediated CaM kinase II translocation to synapses and activation is important in long-term potentiation in hippocampus [13, 25, 26], and spinal cord data showing that CaM kinase II can elicit and mediate sensitization caused by the activator of nociceptive afferent fibers, capsaicin [27, 28]. Accordingly, we found that CCI caused a partial activation of CaM kinase II in spinal cord. This was monitored by ex vivo enzyme assays, as a constitutively active (autophosphorylated, previously activated) form of the enzyme. PSD-95 mutant mice failed to show CCI-induced activation of CaM kinase II, however, suggesting that PSD-95 is essential in assembling an in vivo connection between the NMDA receptor and CaM kinase II under these conditions. Total CaM kinase II activity and immunoreactivity were unaltered by CCI or by the PSD-95 mutation. The fact that a high concentration of NMDA (plus glycine) could readily evoke CaM kinase II activation in PSD-95 mutant ani-

mals while CCI-induced enzyme activation was prevented could suggest that the CCI response involves a particular subpopulation of the enzyme, perhaps assembled in a specific functional arrangement.

An alternative hypothesis could be that the CCI-induced CaM kinase II response, but not that induced by NMDA (plus glycine) alone, might rely on auxiliary regulatory events, for which phosphorylation of the NMDA receptor would be a strong candidate. Although we detected some increase in phosphorylation of NR1-Ser^{897} (a PKA target) in CCI, we could find no evidence for altered association of PKA or AKAP79/150 with spinal NMDA receptor complexes in PSD-95 mutant mice. This argues against a failure of AKAP-mediated localization of kinases in the proximity of the NMDA receptor being the functionally critical deficit in the PSD-95 mutant mice.

In contrast, we found that the levels of overall CaM kinase IIα and phospho-Thr^{286} CaM kinase IIα that could be immunoprecipitated with NR2A/B subunits were clearly increased ipsilateral to CCI in wild-type, but not PSD-95 mutant mice. CaM kinase II is a major component of NMDA receptor complexes [3, 4] and can translocate to synapses upon further NMDA receptor activation [25]. It is known that CaM kinase II can associate directly with NR2A/B subunits, and this interaction may be reduced when PSD-95 is docked to receptor carboxy-tail sequences [29]. Nevertheless, here in a physiological model of neuropathic pain, the injury-induced association of CaM kinase II with the NMDA receptor is shown to be PSD-95 dependent, suggesting that its assembly into multiprotein complexes may be a key factor for in vivo function. Such functional microdomains organized in the vicinity of NMDA receptor complexes [30] may well be critical for neuropathic sensitization. While altered interactions of PSD-95 with other potential partners in the mutant mice may make a contribution to their lack of neuropathic reflex sensitization, the present evidence indicates that disruption of NMDA receptor:PSD-95:CaM kinase II functional microdomains plays a key role.

Supplementary Material

Supplementary material including detailed Experimental Procedures and data on morphological analysis of sciatic nerve fibers is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

This work was supported by the Wellcome Trust (S.M.F.-W., H.H., and S.G.N.G.) and the Medical Research Council (R.M.). Thanks to Louise Anderson and Jane Robinson for technical support and to the Faculty of Veterinary Medicine, University of Edinburgh for the award of a postgraduate studentship to E.M.G. and a summer vacation studentship to J. Bowen.

Received: September 30, 2002
Revised: November 22, 2002
Accepted: December 9, 2002
Published: February 18, 2003

References

1. Mayer, D.J., Mao, J., Holt, J., and Price, D.D. (1999). Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. Proc. Natl. Acad. Sci. USA *96*, 7731–7736.
2. Boyce, S., Wyatt, A., Webb, J.K., O'Donnell, R., Mason, G., Rigby, M., Sirinathsinghji, D., Hill, R.G., and Rupniak, N.M.J. (1999). Selective NMDA NR2B antagonists induce antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. Neuropharmacology *38*, 611–623.
3. Husi, H., and Grant, S.G. (2001). Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. J. Neurochem. *77*, 281–291.
4. Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P., and Grant, S.G. (2000). Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat. Neurosci. *3*, 661–669.
5. Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G.M., Morrison, J.H., et al. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature *396*, 433–439.
6. Bennett, G.J., and Xie, Y.K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain *33*, 87–107.
7. Guilbaud, G., Gautron, M., Jazat, F., Ratinahirana, H., Hassig, R., and Hauw, J.J. (1993). Time-course of degeneration and regeneration of myelinated nerve fibers following chronic loose ligatures of the rat sciatic nerve—can nerve lesions be linked to the abnormal pain-related behaviors. Pain *53*, 147–158.
8. Sommer, C., Laonde, A., Heckman, H.M., Rodriguez, M., and Myers, R.R. (1995). Quantitative neuropathology of a focal nerve injury causing hyperalgesia. J. Neuropathol. Exp. Neurol. *54*, 635–643.
9. Woolf, C.J., and Costigan, M. (1999). Transcriptional and posttranslational plasticity and the generation of inflammatory pain. Proc. Natl. Acad. Sci. USA *96*, 7723–7730.
10. Arner, S., and Meyerson, B.A. (1988). Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. Pain *33*, 11–23.
11. Coderre, T.J., and Melzack, R. (1992). The contribution of excitatory amino-acids to central sensitization and persistent nociception after formalin-induced tissue-injury. J. Neurosci. *12*, 3665–3670.
12. Chaplan, S.R., Malmberg, A.B., and Yaksh, T.L. (1997). Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. J. Pharmacol. Exp. Ther. *280*, 829–838.
13. Gardoni, F., Caputi, A., Cimino, M., Pastorino, L., Cattabeni, F., and Di Luca, M. (1998). Calcium/calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities. J. Neurochem. *71*, 1733–1741.
14. Strack, S., McNeill, R.B., and Colbran, R.J. (2000). Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. J. Biol. Chem. *275*, 23798–23806.
15. Gardoni, F., Schrama, L.H., van Dalen, J.J., Gispen, W.H., Cattabeni, F., and Di Luca, M. (1999). αCaMKII binding to the C-terminal tail of NMDA receptor subunit NR2A and its modulation by autophosphorylation. FEBS Lett. *456*, 394–398.
16. Lisman, J., Malenka, R.C., Nicoll, R.A., and Malinow, R. (1997). Learning mechanisms: the case for CaM-KII. Science *276*, 2001–2002.
17. Bayer, K.U., De Koninck, P., Leonard, A.S., Hell, J.W., and Schulman, H. (2001). Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature *411*, 801–805.
18. Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991). The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem. Biophys. Res. Commun. *181*, 968–975.
19. Gailly, P. (1998). Ca^{2+} entry in CHO cells, after Ca^{2+} stores depletion, is mediated by arachidonic acid. Cell Calcium *24*, 293–304.
20. Tingley, W.G., Ehlers, M.D., Kameyama, K., Doherty, C., Ptak, J.B., Riley, C.T., and Huganir, R.L. (1997). Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J. Biol. Chem. *272*, 5157–5166.
21. Colledge, M., Dean, R.A., Scott, G.K., Langeberg, L.K., Huganir,

R.L., and Scott, J.D. (2000). Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron *27*, 107–119.
22. Siegan, J.B., and Sagen, J. (1995). Attenuation of NMDA-induced spinal hypersensitivity by adrenal medullary transplants. Brain Res. *680*, 88–98.
23. Tao, F., Tao, Y.X., Gonzales, J.A., Fanf, M., Mao, P., and Johns, R.A. (2001). Knockdown of PSD-95/SAP90 delays the development of neuropathic pain in rats. Neuroreport *12*, 3251–3255.
24. Tao, Y., Huang, Y., Mei, L., and Johns, R.A. (2000). Expression of PSD-95/SAP90 is critical for N-methyl-D-aspartate receptor-mediated thermal hyperalgesia in the spinal cord. Neuroscience *98*, 201–206.
25. Shen, K., and Meyer, T. (1999). Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science *284*, 162–166.
26. Fukunaga, K., Rich, D.P., and Soderling, T.R. (1989). Generation of the $Ca^{2(+)}$-independent form of Ca^{2+}/calmodulin-dependent protein kinase II in cerebellar granule cells. J. Biol. Chem. *264*, 21830–21836.
27. Fang, L., Wu, J., Lin, Q., and Willis, W.D. (2002). Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J. Neurosci. *22*, 4196–4204.
28. Kolaj, M., Cerne, R., Cheng, G., Brickey, D.A., and Randic, M. (1994). Alpha subunit of calcium/calmodulin-dependent protein kinase enhances excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. J. Neurophysiol. *72*, 2525–2531.
29. Gardoni, F., Schrama, L.H., Kamel, A., Gespen, W.H., Cattabeni, F., and Di Luca, M. (2001). Hippocampal synaptic plasticity involves competition between Ca^{2+}/calmodulin-dependent protein kinase II and postsynaptic density 95 for binding to the NR2A subunit of the NMDA receptor. J. Neurosci. *21*, 1501–1509.
30. Zhabotinsky, A.M. (2000). Bistability in the $Ca(^{2+})$/calmodulin-dependent protein kinase-phosphatase system. Biophys. J. *79*, 2211–2221.