PDZ Domains at Excitatory Synapses: Potential Molecular Targets for Persistent Pain Treatment

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Abstract: Persistent pain, a common clinical condition, could be caused by inflammation, tissue injury secondary to trauma or surgery, and nerve injuries. It is often inadequately controlled by current treatments, such as opioids and non-steroidal anti-inflammatory drugs. The PDZ (Postsynaptic density 95, Discs large, and Zonula occludens-1) domains are ubiquitous protein interaction modules often found among multi-protein signaling complexes at neuronal synapses. Recent preclinical research shows that targeted disruption of PDZ domain-mediated protein interaction among *N*-methyl-D-aspartate (NMDA) receptor signaling complexes significantly attenuates the development and maintenance of persistent pain without affecting nociceptive responsiveness to acute pain. PDZ domains at excitatory synapses may be new molecular targets for prevention and treatment of persistent pain. Here, we illustrate expression and distribution of the PDZ domain-containing proteins associated with NMDA receptors in the pain-related regions of the central nervous system, review the evidence for their roles in persistent pain states, and discuss potential mechanisms by which these PDZ domain-containing proteins are involved in persistent pain.

Key Words: PSD-93, PSD-95, NMDA receptors, AMPA receptors, Trafficking, Spinal cord, Persistent pain, Chronic pain.

INTRODUCTION

Protein-protein interactions govern many critical physiologic and pathologic processes, such as cell growth, intercellular communication, learning and memory, cell death, stroke, and chronic pain, through dynamic interactions between modular protein domains and their cognate binding partners [2, 41, 50]. The PDZ (Postsynaptic density 95, Discs large, and Zonula occludens-1) domains are modular protein interaction domains often found to bind to short peptide motifs at the extreme carboxy (C) terminals of other proteins [14, 23], although they can also have other modes of interaction. In the mammalian central nervous system, C-terminal motifs of *N*-methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B bind to PSD-93/chapsyn (channel-associated protein of synapses)-110 [5, 22], postsynaptic density (PSD)-95/synaptic-associated protein (SAP) 90 [11, 25], and SAP102 through PDZ domain-mediated protein interactions [27, 36]. These PDZ proteins not only are involved in synaptic NMDA receptor trafficking but also couple the NMDA receptors to intracellular proteins and signaling enzymes [7, 45, 59]. Recent studies indicate that disruption of the PDZ domain-mediated protein interaction between the NMDA receptor subunits NR2A/2B and PSD-93 or PSD-95 significantly attenuates nerve injury- and tissue injury-induced persistent pain [17, 51], suggesting that the PDZ domains at excitatory synapses may be new molecular targets for persistent pain treatment. Here, we illustrate the expression and distribution of two NMDA receptor-interacting PDZ proteins, PSD-93 and PSD-95, in pain-related regions of the nervous system, review the evidence for their roles in persistent pain states, and discuss potential mechanisms by which these proteins are involved in persistent pain.

NMDA RECEPTOR-INTERACTING PDZ PROTEINS

The NMDA receptor-interacting PDZ proteins include PSD-93 [5, 21], PSD-95 [11, 25], and SAP102 [27, 36]. The NMDA receptors are formed by the combination of a common NR1 subunit and at least one or more of four different NR2 subunits, NR2A–2D [35, 38]. The first and second PDZ domains of PSD-93, PSD-95, and SAP102 interact with long cytoplasmic C-terminal motifs of NR2A and NR2B subunits. These NMDA receptor-interacting PDZ proteins are identified to have structural similarity with another PDZ domain-containing protein, SAP97/hdlg [29, 37]. They are generically referred to as membrane-associated guanylate kinases (MAGUKs) and contain three tandem PDZ domains (PDZ1–3) at the N-terminal side, an Src homology region 3 (SH3) domain in the middle, and a guanylate kinase-like (GK) domain at the C-terminal end (Fig. 1). PDZ domains are motifs of ~90 amino acid repeats. In addition to binding to the C-termini of NR2A and NR2B subunits, the second PDZ domain of PSD-95 and PSD-93 also form heterodimeric PDZ-PDZ interactions with the PDZ domain of neuronal nitric oxide synthase (nNOS) [4, 5, 22]. Three PDZ domains of PSD-95 and PSD-93 also bind to other intracellular proteins such as synaptic GTPase-activating protein (SynGAP) [9, 24, 26] and Src family of proteins [55] (Fig. 2A). X-ray crystallography has revealed that the second PDZ domain of PSD-95 contains a peptide-binding groove on the surface that consists of a β B strand and an α B helix [15, 56]. The hydrophobic groove is a binding pocket for the C-terminal motifs of NR2A and NR2B and for a β -finger peptide from the nNOS PDZ domain [15, 56, 57].

The other domains of PSD-93 and PSD-95 also appear to be involved in protein-protein interactions. For example, the guanylate kinase domain of PSD-93 does not have any enzymatic activity, but it specifically binds to the microtubule-associated protein 1A (MAP1A) [6], a major constituent of neuronal microtubules that plays a central role in neuronal

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Fig. (1). Diagrammatic representation of structure of membrane-associated guanylate kinases (MAGUKs). MAGUKs include PSD-93/chapsyn-110, PSD-95/SAP90, SAP102, and SAP97/hdlg. They are identified to have structural similarity with three tandem PDZ domains (PDZ1-3) at the N-terminal side, an Src homology region 3 (SH3) domain in the middle, and a guanylate kinase-like (GK) domain at the C-terminal end.

morphogenesis [31]. The SH3 domain of PSD-93 or PSD-95 is found to interact with the guanylate kinase domain in an intramolecular or intermolecular manner [47]. Thus, PSD-93 and PSD-95 serve as adaptor proteins to form large synaptic macromolecular complexes that help to organize synaptic structure.

EXPRESSION AND DISTRIBUTION OF PSD-93 AND PSD-95 IN CENTRAL PAIN-RELATED REGIONS

Messenger RNAs and proteins of PSD-93 and PSD-95 are expressed highly in some pain-related regions of the nervous system. RNA extracted from the dorsal root ganglion, spinal cord, and forebrain was probed using reverse transcriptase-polymerase chain reaction (PCR) analysis. The PCR products of PSD-93 and PSD-95 were detected in high concentration in the spinal cord (especially in the dorsal

horn) and in forebrain areas [50, 52]. In contrast, they were weakly detected or not at all in the dorsal root ganglion [50, 52]. The PCR products were then directly cloned into the pCR2.1-TOPO vector and verified as PSD-93 and PSD-95 by automatic DNA sequencing. Immunoblot analysis further revealed abundant protein expression of PSD-93 and PSD-95 in the dorsal horn of the spinal cord and in forebrain areas, but not in the ventral horn of the spinal cord or dorsal root ganglion [50, 52, 65]. Using immunocytochemistry, we found that their immunoreactivities occurred at a higher density in the superficial laminae and at a lower density in other laminae of the spinal dorsal horn [50, 52, 65]. Under electron microscopy, the subcellular localization of PSD-93 has been characterized. In sections of the superficial dorsal horn or the anterior cingulate cortex (ACC) of forebrain, immunogold labeling with a PSD-93 antibody was associated with the

Fig. (2). The proposed potential molecular mechanisms by which PSD-93 knockout or PSD-95 mutation produces antinociception during persistent pain states. In wildtype (WT) mice (A), PSD-95 and PSD-93 may facilitate the functional coupling between the NMDA receptors and CaMKII. In addition, PSD-95 and PSD-93 couple the NMDA receptors to other intracellular signal pathways. For example, the second PDZ domain of PSD-95 or PSD-93 binds to both NR2A/2B and nNOS. The third PDZ domain of PSD-95 or PSD-93 interacts with an intercellular protein neuroligin and intracellular proteins, such as SynGAP and the Src family of proteins. PSD-95 and PSD-93 also couples the NMDAR complex to the mGluR-Homer-Shank complex *via* interaction of their GK domain with GK associated protein (GKAP). In PSD-93 knockout mice (B), the deletion of three PDZ domains and downstream sequences of PSD-93 not only reduces synaptic expression and function of the NMDA receptors, but it might also dissociate the NMDA receptors from some intracellular signaling pathways (e.g. NO signaling, Ras signaling, the Src family of proteins, and the mGluR-Homer-Shank complex). In PSD-95 mutant mice (C), the deletion of the third PDZ domain and downstream sequences of PSD-95 not only prevents PSD-95-mediated facilitation of the functional coupling of NMDA receptors to CaMKII, but it might also dissociate the NMDA receptors from other intracellular signaling pathways (e.g. Ras signaling, the Src family of proteins, and the mGluR-Homer-Shank complex), although the PSD-95 might still couple the NMDA receptor complex to NO signaling and synaptic expression and function of the NMDA receptors are intact.

postsynaptic membrane in neuronal synapses. The superficial dorsal horn and ACC are important sites for processing noxious stimulation in the central nervous system [44]. The area-specific expression and distribution of PSD-93 and PSD-95 in these two pain-related regions suggest that they might have important implications for the mechanisms of central nociceptive processing.

Interestingly, PSD-93 has distinct expression and distribution patterns in the superficial dorsal horn, compared to PSD-95, although both of them have been identified at glutamatergic synapses [50, 52, 65]. PSD-93 is expressed mainly in laminae I and II and outer lamina III [52, 65], whereas PSD-95 is distributed predominantly in lamina I and outer lamina II [50]. The postsynaptic neurons in inner lamina II differ considerably from those in lamina I and outer lamina II with respect to forming synaptic architecture with the primary afferent terminals [8, 20]. Compared with PSD-95, PSD-93 seems to have unique expression patterns in the inner lamina II.

EFFECT OF TARGETED DISRUPTION OF THE PSD-93 OR PSD-95 GENE ON PERSISTENT PAIN

PSD-95 was the first NMDA receptor-interacting PDZ protein that was reported to be required for NMDA receptor-mediated sensitization of nociceptive behavioral reflexes [48]. Spinal PSD-95 knockdown attenuated NMDA-triggered facilitation of the tail-flick reflex in response to heat stimulation and reduced nerve injury-induced mechanical and thermal pain hypersensitivity during both the development and maintenance of chronic neuropathic pain [50, 53, 54]. Garry *et al.* further reported that PSD-95 mutant mice displayed a complete lack of reflex sensitization to mechanical, thermal, and cold nociceptive stimuli following the chronic constriction of the sciatic nerve [18]. Similarly, spinal PSD-93 knockdown in rats or PSD-93 knockout in mice prevented NMDA receptor-dependent persistent or chronic pain from spinal nerve injury or injection of complete Freund's adjuvant [52, 65]. It bears noting that spinal PSD-93 or PSD-95 knockdown did not change basal responses to mechanical and thermal stimuli and motor function [50, 53, 54, 65]. No significant differences in paw withdrawal latencies (in response to thermal stimulation) or frequencies (in response to mechanical stimulation) were observed among wildtype, heterozygous, PSD-93 knockout or PSD-95 mutant mice [18, 52]. These genetic knockout or mutant mice also have normal appearance and locomotor activity [18, 32, 52]. Thus, the targeted disruption of the PSD-93 or PSD-95 gene only reduces NMDA receptor-dependent central sensitization of behavioral reflexes during persistent or chronic pain states without affecting acute nociceptive transmission.

POTENTIAL MOLECULAR MECHANISM OF PSD-93 AND PSD-95 ACTION IN PERSISTENT PAIN

PSD-93 or PSD-95 deletion may alter synaptic NMDA receptor expression and function, which, in turn, results in impaired NMDA receptor-dependent persistent pain. It is well documented that the development and maintenance of chronic or persistent pain are dependent on synaptic NMDA receptor expression and activation [40, 44]. The NR2 subunit determines synaptic localization and function of the NMDA

receptors, as deletion of the C-terminal tail of NR2 results in impaired NMDA receptor-mediated synaptic activity [13, 49]. As discussed above, C-terminal tails of the NR2A and NR2B subunits directly bind to the first and second PDZ domains of PSD-93 and PSD-95 [5, 11, 22, 25]. *In vitro* studies showed that PSD-95 enhanced NMDA receptor clustering at synapses [43] and inhibited NR2B-mediated internalization [48]. Co-expression of PSD-95 with the NMDA receptor increases surface expression of the NMDA receptors and enhances synaptic NMDA receptor function [28]. These *in vitro* findings indicate that PSD-93 and PSD-95, as molecular scaffold proteins, may cluster and bind to the NMDA receptors at synaptic membranes and modulate their synaptic function. In the spinal dorsal horn, the distribution of PSD-93 or PSD-95 overlaps that of the NMDA receptors [50, 52]. In sections from the superficial dorsal horn and the ACC labeled with both PSD-93 and NR2A/2B antibodies, neuronal synapses showed labeling for both antibodies interspersed along the postsynaptic membrane [52]. Co-immunoprecipitation further showed that NR2A and NR2B antibodies were able to immunoprecipitate themselves, as well as PSD-93 and PSD-95, in the postsynaptic density fractions from the dorsal horn and forebrain [50, 52]. Interaction of PSD-93 or PSD-95 with the NMDA receptors at the synapses in these pain-related regions suggests that PSD-93 and PSD-95 might be required for synaptic NMDA receptor expression and function. Indeed, PSD-93 knockout blunted NMDA receptor-mediated excitatory postsynaptic currents and potentials in the neurons of superficial dorsal horn, ACC, and insular cortex [52]. PSD-93 deletion also reduced surface NR2A and NR2B expression in the dorsal horn neurons [52] (Fig. 2B). It appears that the first and second PDZ domains of PSD-93 might be important for NMDA receptor synaptic targeting and function.

The second PDZ domain of PSD-93 also binds to intracellular proteins [6, 51], such as neuronal nitric oxide (NO) synthase (nNOS). It is reasonable to conclude that PSD-93 deletion also dissociates synaptic NMDA receptors from downstream signaling pathways. This hypothesis is supported by our studies showing that PSD-93 knockout in cortical neurons prevented NMDA receptor/NO-dependent neuronal cell death caused by platelet-activating factor [60, 61]. Thus, the disruption of PDZ domain-mediated protein interaction between PSD-93 and NR2A or NR2B through PSD-93 deletion results in blunted NMDA receptor-dependent persistent pain, possibly by the mechanisms of (1) alteration of synaptic NMDA receptor expression and function (Fig. 2B and 3C), and (2) dissociation of synaptic NMDA receptors from downstream signaling pathways (Fig. 2B and 3C). PDZ domains at excitatory synapses may be effective biochemical targets for the prevention and treatment of persistent or chronic pain.

Interestingly, PSD-95 mutant mice exhibited normal synaptic NMDA receptor expression and NMDA receptor-mediated excitatory postsynaptic currents in hippocampal neurons, although they displayed altered long-term potentiation and impaired learning and neuropathic pain [18, 34]. Does this suggest that PSD-93 and PSD-95 are involved in central sensitization of the NMDA receptor-dependent chronic or persistent pain through distinct mechanisms? Before this ques-

tion is answered, it should be noted that the PSD-93 knockout mice and the PSD-95 mutant mice can be differentiated by the PDZ domains that are targeted for disruption. The coding sequence of three PDZ domains of PSD-93 is completely deleted in PSD-93 knockout mice [32], whereas PSD-95 transgenic mice carry a targeted mutation in the PSD-95 gene that leaves the first two PDZ domains intact by introducing a stop codon into the third PDZ domain and replacing downstream sequences with an internal ribosome entry site [34] (Fig. 2C). As discussed above, the first and second PDZ domains of PSD-93 and PSD-95 are critical for binding and anchoring of the NMDA receptors at synaptic membrane [28, 43, 48]. That the first two PDZ domains were not detected in synaptosome subfractions of PSD-95 mutant mice might be related to the specificity of the antibody, as this antibody also did not detect the first two PDZ domains of full-length PSD-95 in synaptosome subfractions of wild-type mice [34]. Thus, with the use of a suitable antibody, the first two PDZ domains may be detected in synaptosome subfractions of PSD-95-mutant mice (Fig. 2C). These two PDZ domains may be involved in maintaining normal synaptic localization and postsynaptic function of the NMDA receptors in PSD-95 mutant mice (Fig. 2C). PSD-95 may have similar effects as PSD-93 on synaptic NMDA receptor surface expression and function in central neurons if the first two PDZ domains of PSD-95 are completely knocked out. Further studies are required to address this issue using the PSD-95 knockout mice with the complete deletion of the coding sequence of PSD-95's three PDZ domains.

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is essential for the NMDA receptor-dependent spinal sensitization of behavioral reflexes in neuropathic pain [16, 18]. PSD-95 facilitated the functional coupling between the NMDA receptors and CaMKII in a neuropathic pain model [18]. Moreover, nerve injury-induced increases in CaMKII activity and in CaMKII association with NR2 were prevented in PSD-95 mutant mice [18]. Thus, the blunted neuropathic pain in PSD-95 mutant mice might be due to impaired interaction of CaMKII with the NMDA receptors (Fig. 2C). It should be noted that the third PDZ domain of PSD-95 also binds to other intracellular proteins, such as SynGAP [9, 24] and the Src family of proteins [55] (Fig. 2A). *Via* its GK domain interaction with GK-associated protein (GKAP), PSD-95 couples the NMDA receptor complex to the metabotropic glutamate receptor (mGluR)-Homer-Shank complex [57] (Fig. 2A). It might be possible that the dissociation of the NMDA receptors with other intracellular signaling pathways in PSD-95 mutant mice may cause the impaired neuropathic pain (Fig. 2C).

The genetic knockout and mutant models are very useful, especially when direct antagonists are unavailable. However, these models may be of limited value by themselves because compensatory mechanisms could interfere in interpretation of the results. To further confirm the functional roles of spinal PSD-93 and PSD-95 in persistent or chronic pain, we transiently knocked down the expression of spinal cord PSD-93 or PSD-95 using the antisense oligodeoxynucleotide (AS ODN) strategy. Consistent with the genetic knockout and mutant models [18, 52], intrathecal administration of PSD-93 or PSD-95 AS ODNs, but not of the corresponding

sense and missense ODNs, attenuated the development and maintenance of mechanical and thermal pain hypersensitivity during chronic inflammatory and neuropathic pain states [50, 53, 54, 65]. However, PSD-93 and PSD-95 AS ODNs did not significantly alter the expression of the NMDA receptors in the membrane fraction of the spinal cord [50, 65].

What is the underlying mechanism by which acute transient deficiency of PSD-93 or PSD-95 protein affects chronic pain? It is noteworthy that PSD-93 and PSD-95 also function as the scaffolding proteins to assemble a specific set of signaling proteins around the NMDA receptors [51]. These signaling proteins, including nNOS as mentioned above, participate in downstream signaling by the NMDA receptors [6]. It is very likely that the acute transient deficiency of spinal cord PSD-93 or PSD-95 produces antinociception during chronic pain by dissociating the NMDA receptors from downstream signaling pathways (Fig. 3). This view is supported by other studies that show that acute transient deficiency of PSD-95 or perturbation of NMDA receptor-PSD-95 protein interaction significantly attenuated excitotoxicity, brain damage, and NO production that was triggered by the NMDA receptors selectively, without affecting the expression and function of the NMDA receptors in the central neurons [1, 46]. That nNOS specifically couples to the NMDA receptor complex *via* PSD-93 and PSD-95 through PDZ domain-mediated protein interaction occurs in the spinal cord neurons [50, 52]. The functional role of spinal cord nNOS and NO in chronic inflammatory pain has been demonstrated [12], although conflicting pharmacologic evidence has been reported regarding the effects of systemic or spinal treatment with specific or non-specific NOS inhibitors on neuropathic pain [30, 33, 63, 64]. It seems that the mechanism by which antinociception is caused by acute transient deficiency of spinal PSD-93 or PSD-95 during chronic pain may be related to disruption of the coupling of nNOS to the NMDA receptor and to blockage of NO production triggered *via* NMDA receptor activation.

In that PSD-93 and PSD-95 also bind to other postsynaptic membrane proteins (e.g., potassium channels [5, 22] and δ_2 glutamate receptors [42]) and intracellular proteins (e.g., the microtubule-associated protein 1A [6] and SynGAP [9, 24, 26]), the detailed mechanism by which acute transient deficiency of spinal PSD-93 or PSD-95 affects spinal central sensitization during chronic pain remains to be explored.

CONCLUSION

Persistent pain, particularly nerve injury-induced neuropathic pain, is poorly managed by current therapies, such as opioids and non-steroidal anti-inflammatory drugs. The antagonists of the glutamate receptors, including NMDA receptors or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, are effective in reducing pain hypersensitivity in animal models and clinical settings, but produce unacceptable side effects, such as psychotomimesis, ataxia, sedation, etc [9]. Similarly, the targeted disruption of the proteins that bind to various glutamate receptor subunits (e.g. stargazin interacts with the AMPA receptor subunits GluR1, 2, and 4) is not a good option for pharmacologic intervention, because the disruption of the interaction between these proteins and their binding receptor subunits may lead

Fig. (3). The proposed potential molecular mechanisms by which acute transient deficiency (knockdown) and chronic congenital deficiency (knockout) of PSD-93 produce antinociception during persistent pain states. In normal animals (**A**), PSD-93 and PSD-95 cluster and target the NMDA receptors at synapses and couple the intracellular signal proteins (such as nNOS) to synaptic NMDA receptors, mediating central pain signal transmission. In the PSD-93 antisense (AS) ODN-treated groups (**B**), acute knockdown of PSD-93 expression does not alter membrane NMDA receptor expression, but partially dissociates the NMDA receptors from intracellular signaling (such as nNOS), resulting in impaired pain hypersensitivity during persistent or chronic pain states. In contrast, PSD-93 deletion in the PSD-93 knockout (KO) mice (**C**) reduces surface NR2A and NR2B expression, decreases synaptic NMDA receptor functions, and dissociates the NMDA receptors from the downstream signaling (such as NO), resulting in blunted pain hypersensitivity during persistent or chronic pain states. Neither PSD-93 knockdown nor PSD-93 knockout alters PSD-95 expression in postsynaptic density.

to similar side effects as those of the glutamate receptor antagonists. For example, stargazin deletion results in epilepsy, ataxia, and abnormal motor behaviors [21, 39]. Thus, the development of the glutamate receptor subunit- and site-specific drugs holds promise for the development of new strategies for precise and selective therapeutic intervention of persistent pain with or without reducing side effects [62]. As discussed above, the disruption of the PDZ domain-mediated protein interaction between NMDA receptor subunits NR2A/2B and PSD-93 or PSD-95 through genetic targeting PSD-93 or PSD-95 significantly attenuates tissue injury- and nerve injury-induced persistent pain, with preservation of acute pain transmission [50-54, 65]. These findings indicate that PDZ domain-mediated protein interaction at excitatory synapses might be new molecular targets for prevention and treatment of persistent pain. However, the genetic approaches (knockout and knockdown models) are therapeutically impractical in a clinical setting. A previous study reported that perturbing NMDA receptor-PSD-95 protein interactions attenuated ischemic brain damage by introducing the inhibitory peptides that encode the C-terminus of NR2A/NR2B or the second PDZ domain of PSD-95 [1]. Moreover, the disruption of the PDZ domain-mediated protein interaction between AMPA receptor subunit GluR2 and its binding partners [such as protein interacting with C-kinase 1 (PICK1)] by the inhibitory peptides that encode C-terminus of GluR2 has recently been shown to produce antinociceptive effects in nerve injury-induced persistent pain without affecting nociceptive responsiveness to acute pain [19]. It is very likely that these inhibitory peptides might be clinically therapeutically practical and highly novel therapies for prevention or treatment of persistent pain. Studies to further determine the effects of these inhibitory peptides on persistent pain through their perturbation of the PDZ domain-mediated protein interaction at excitatory synapses are required.

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