AMPA receptor–PDZ interactions in facilitation of spinal sensory synapses

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Silent synapses form between some primary sensory afferents and dorsal horn neurons in the spinal cord. Molecular mechanisms for activation or conversion of silent synapses to conducting synapses are unknown. Serotonin can trigger activation of silent synapses in dorsal horn neurons by recruiting AMPA receptors. AMPA-receptor subunits GluR2 and GluR3 interact via their cytoplasmic *C* termini with PDZ-domain-containing proteins such as GRIP (glutamate receptor interacting protein), but the functional significance of these interactions is unclear. Here we demonstrate that protein interactions involving the GluR2/3 *C* terminus are important for serotonin-induced activation of silent synapses in the spinal cord. Furthermore, PKC is a necessary and sufficient trigger for this activation. These results implicate AMPA receptor–PDZ interactions in mechanisms underlying sensory synaptic potentiation and provide insights into the pathogenesis of chronic pain.

At central neuronal synapses, neurotransmitter receptors are concentrated in the postsynaptic membrane, and regulation of the distribution and density of these receptors may play a critical role in synaptic plasticity[1–3]. Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), acts on three major classes of glutamate receptors: AMPA/kainate, NMDA and metabotropic glutamate receptors[4]. In excitatory synapses between sensory afferent fibers and dorsal horn neurons that convey sensory inputs into the spinal cord[5], the AMPA subclass of ionotropic glutamate receptors mediates most fast synaptic transmission[6,7].

AMPA receptors are formed by heteromeric combinations of four subunits, GluR1–4 (ref. 4). AMPA receptor subunits GluR2 and GluR3 share a common cytoplasmic *C*-terminal sequence (the last four amino acids being -SVKI) that interacts with PDZ- domain-containing proteins such as GRIP (glutamate receptor interacting protein)[8], ABP (AMPA receptor binding protein)[9] and PICK1 (protein interacting with C kinase)[10]. GRIP and ABP are closely homologous proteins, and we use the term GRIP/ABP to include both proteins. Interference with GluR2/3–PDZ interactions by dominant negative approaches inhibits the synaptic clustering of AMPA receptors[8]; however, the importance of these interactions for synaptic function and plasticity is unknown.

Sensory synaptic transmission in the spinal cord dorsal horn is subject to descending facilitation and inhibition from supraspinal structures, including the rostral ventromedial medulla (RVM), which relays signals from the periaqueductal gray to dorsal horn sensory neurons[11]. Many RVM-spinal projecting neurons are serotonergic, and serotonin (5-HT) released within the spinal cord modulates nociceptive transmission biphasically. Whereas the inhibitory effects of 5-HT are generally short-lasting and important for acute antinociception induced by focal electrical stimulation[12], the facilitatory effects of 5-HT, mediated by distinct subtypes of 5-HT receptors[13,14], could contribute to chronic pain after tissue injury[15,16]. Serotonin's facilitatory effect is mediated, at least in part, by the activation of silent glutamatergic synapses on spinal dorsal horn sensory neurons[14]. The intracellular pathways underlying this activation have not been investigated.

Here we show that interactions between GluR2/3 and proteins recognizing its *C*-terminal PDZ-binding motif are required for the 5-HT-induced activation of silent sensory synapses in spinal cord slices. Postsynaptic activation of protein kinase C (PKC) is a necessary and sufficient trigger for synaptic potentiation by 5-HT; moreover, the PKC-mediated activation of silent synapses is also dependent on GluR2/3–PDZ interactions. These findings outline an intracellular pathway for the activation of silent glutamatergic synapses in dorsal horn neurons.

Results

GluR2/3 and GRIP, a protein with seven PDZ domains that binds specifically to the *C* terminus of GluR2/3, are expressed in the spinal cord (**Fig. 1a**); immunostaining revealed coexistence of GluR2/3 and GRIP in many dorsal horn neurons (data not shown). Long-term overexpression of the *C* terminus of GluR2 in hippocampal neurons reduces the number of synaptic AMPA receptor clusters[8], suggesting that interaction between GluR2/3 and PDZ proteins is involved in the postsynaptic targeting of AMPA receptors. To examine the functional significance of GluR2/3–PDZ interactions in sensory synaptic transmission, we

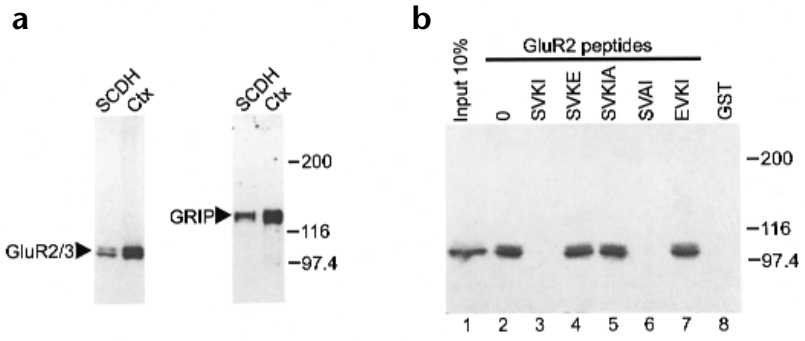

Fig. 1. Expression of GluR2/3 and GRIP in spinal cord dorsal horn neurons. (**a**) Western blot for GluR2/3 and GRIP in homogenates of spinal cord dorsal horn (SCDH) and cerebral cortex (10 μg protein loaded in each lane). (**b**) GST-pulldown assay for GluR2 binding to GRIP in the presence of competing peptides (as indicated). Extracts of COS cells expressing GluR2 were incubated with GST-GRIP immobilized on glutathione beads, and bound GluR2 was detected by immunoblotting. GluR2-SVKI and -SVAI (but not GluR2-SVKE, -SVKIA or -EVKI) blocked GluR2 binding to GST-GRIP. GST alone showed no binding of GluR2 (lane 8). Input lane contains 10% of GluR2-containing extract used in the GST-pulldown reactions.

made a synthetic peptide corresponding to the last ten amino acids of GluR2 ('GluR2-SVKI': NVYGIESVKI), which disrupted binding of GluR2 to GRIP (**Fig. 1b**). It was expected that the GluR2-SVKI peptide should also interfere with interactions between GluR2 and other PDZ proteins such as ABP and PICK1. Furthermore, since the last ten amino acids of GluR2 and GluR3 are nearly identical, GluR2-SVKI would also be expected to inhibit GluR3 binding to the same set of PDZ proteins. A control peptide (GluR2-SVKE), in which the PDZ interaction motif was destroyed by substituting the last amino acid (isoleucine) with glutamate, did not interfere with GluR2 binding to GRIP (**Fig. 1b**). Peptides were applied through the patch recording electrode into postsynaptic neurons to examine their effects on synaptic transmission. In spinal cord slices, GluR2-SVKI at 5 μM (n = 5, data not shown) or 50 μM (n = 8) and GluR2-SVKE at 50 μM (n = 5) did not affect basal synaptic responses of dorsal horn neurons (holding potential, –70 mV) to afferent stimulation at the dorsal-root entry zone (DREZ; **Fig. 2a** and **b**). Likewise, injection of GluR2-SVKI into cultured spinal dorsal horn neurons did not change peak whole-cell currents induced by application of 500 μM glutamate (n = 5, **Fig. 2c** and **d**). Thus, the peptides had no effect on baseline synaptic transmission over a time course of half an hour.

Although GluR2-SVKI had no effect on baseline synaptic transmission, it could interfere with the plasticity of AMPA receptor-mediated synaptic responses. The major transmitter for descending RVM-spinal fibers, 5-HT, modulates spinal sensory signaling[12]. When applied to spinal cord slices at 5 μM, 5-HT enhances synaptic transmission between sensory afferent fibers and dorsal horn neurons by activating silent synapses[14]. A possible mechanism for this activation is the functional recruitment or insertion of AMPA receptors into the postsynaptic membrane, a process that might be regulated by protein–protein interactions involving the cytoplasmic tails of AMPA-receptor subunits. We tested if the interaction between the GluR2/3 *C* terminus and its specific PDZ binding partners in postsynaptic neurons is important for 5-HT-induced synaptic facilitation. Indeed, the facilitatory effect of 5-HT (5 μM) was blocked by the presence of 50 μM GluR2-SVKI (n = 8), but not by the same concentration of the control peptide GluR2-SVKE (n = 6; **Fig. 3**), in the patch pipet. In dorsal horn neurons with no exogenous peptide present, 5-HT modulates synaptic transmission biphasically, enhancing at a low dose (5 μM) and inhibiting at a higher dose (50 μM) by acting through different 5-HT receptor subtypes[14]. Whereas 50 μM GluR2-SVKI abolished the facilitatory effect of 5 μM 5-HT, neither this peptide nor 50 μM GluR2-SVKE blocked the inhibition produced by 50 μM 5-HT (n = 3; data not shown). Thus, interfering with GluR2/3 *C*-terminal interactions selectively blocked the intracellular processes underlying facilitation but not inhibition of synaptic transmission by 5-HT.

Spinal sensory transmission is also modulated by acetylcholine (ACh), which is probably released from spinal dorsal horn interneurons[17,18]. Bath application of the muscarinic ACh-receptor agonist carbachol to spinal cord slices produced a dose-dependent inhibition of EPSCs (n = 3–6 for each dose; data not shown). Postsynaptic injection of 50 μM GluR2-SVKI (n = 3) or 50 μM

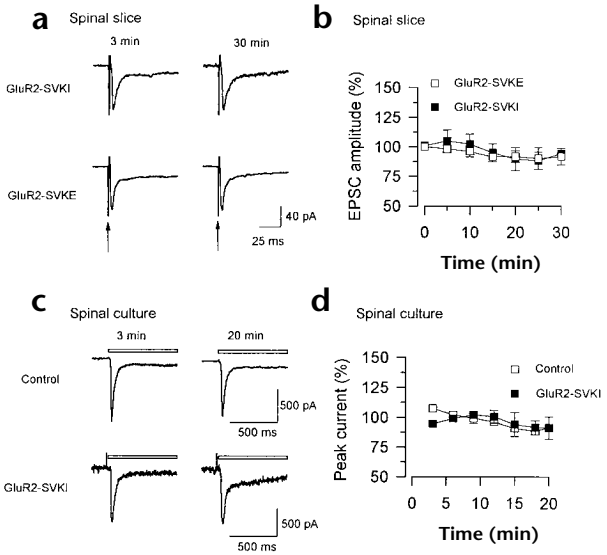

Fig. 2. GluR2-SVKI and GluR2-SVKE peptides do not affect baseline AMPA receptor-mediated responses in spinal cord dorsal horn neurons. (**a–b**) Baseline EPSC amplitudes evoked in dorsal horn neurons from spinal cord slices were not affected over 30 min by the intracellular application of 50 μM GluR2-SVKI (n = 8) or 50 μM GluR2-SVKE (n = 5), as demonstrated by representative traces (**a**) and pooled data (**b**). (**c–d**) The peak amplitudes of AMPA receptor-mediated whole-cell currents induced by 500 μM glutamate (with 2 μM MK-801 to block NMDA receptor-mediated responses) in cultured spinal dorsal horn neurons were not affected over time either in the presence (n = 5) or absence (n = 3) of GluR2-SVKI (50 μM) in the recording pipet. Glutamate application is indicated in representative traces (**c**) by open bars, and time is measured from the point of break-in.

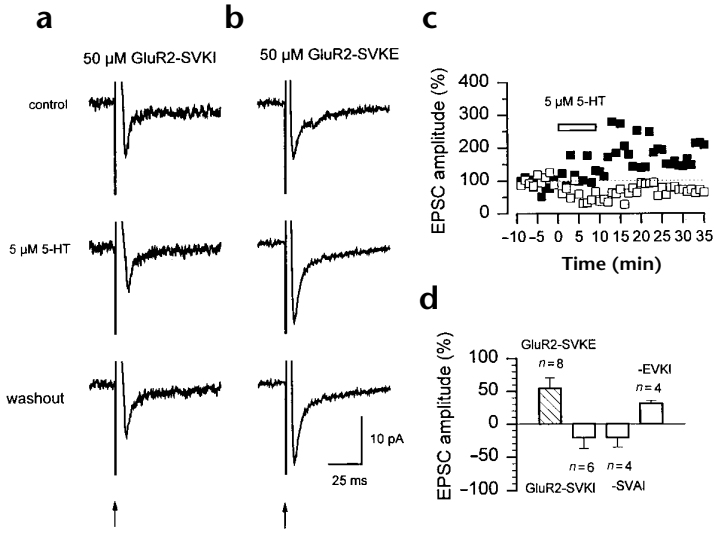

Fig. 3. GluR2-SVKI selectively blocks synaptic facilitation induced by 5-HT. 50 μM GluR2-SVKI (**a**), but not 50 μM GluR2-SVKE (**b**), prevented the facilitation of evoked EPSCs by 5 μM 5-HT in dorsal horn neurons, as shown by representative traces. (**c**) The time course of 5-HT-induced changes in EPSC amplitude is illustrated for the same cells (**a**, **b**), with open squares for responses of the neuron loaded with GluR2-SVKI and closed squares for those of a neuron loaded with GluR2-SVKE. (**d**) Pooled data for experiments as in (**a–c**) demonstrate the ability of 5 μM 5-HT to facilitate EPSCs in the presence of GluR2-SVKE or GluR2-EVKI, but not GluR2-SVKI or GluR2-SVAI, in the recording pipet.

GluR2-SVKE (n = 5) did not block the inhibition of synaptic responses by 20 μM carbachol. These results further demonstrate the specificity of the effect of GluR2-SVKI on synaptic facilitation mediated by 5-HT.

In the spinal cord, PKC is crucial for the induction of chronic pain[19], and activation of PKC enhances sensory synaptic transmission in the dorsal horn, sensitizing the responses of ascending projection neurons to noxious peripheral stimuli[13,20,21]. Because PKC acts downstream of 5-HT$_2$ receptors[22], the receptors critical for the facilitatory effect of low-dose 5-HT[14], we tested whether PKC in postsynaptic neurons was required for synaptic enhancement by 5-HT. When the PKC peptide inhibitor PKCI (19–36; ref. 23) was present in the patch pipet (20 μM), application of 5 μM 5-HT caused depression rather than enhancement of EPSCs (n = 5; 40 ± 13% of peak control responses, **Fig. 4b**), an observation that could reflect unmasking of the synaptic inhibition normally observed only with higher 5-HT concentrations. Bath application of the PKC activator phorbol 12,13-dibutyrate (PDBu; 0.5 μM) enhances postsynaptic responses in spinal dorsal horn neurons[13]. We confirmed synaptic facilitation by PDBu, and showed that it persisted with the control peptide GluR2-SVKE in the recording electrode (n = 7; **Fig. 4d**). However, this potentiation by PDBu was abolished by loading postsynaptic neurons with GluR2-SVKI (n = 6, **Fig. 4e**) or PKCI (n = 5, **Fig. 4c**), suggesting that synaptic facilitation mediated by PKC activation is similar to that produced by 5-HT in its dependence on GluR2/3 *C*-terminal interactions.

Because the GluR2-SVKI peptide contains a sequence (-SVK) that conforms to a PKC phosphorylation consensus (S/T-X-K/R), we were concerned that the peptide might have acted as a competitive inhibitor of PKC catalytic function rather than of GluR2–PDZ interactions. We therefore tested a different control peptide (GluR2-SVAI), in which the basic residue at the –1 position was mutated to alanine, thereby removing the PKC recognition consensus. Like GluR2-SVKI, GluR2-SVAI disrupted GluR2 binding to GRIP (**Fig. 1b**), and postsynaptic injection of GluR2-SVAI (50 μM) prevented 5-HT-induced synaptic facilitation (n = 4; **Fig. 3d**). This suggests that GluR2-SVKI inhibits 5-HT synaptic facilitation by disrupting GluR2–PDZ interactions, rather than acting as a PKC substrate.

In a further control peptide (GluR2-EVKI), the serine at the –3 position was replaced with glutamate. GluR2-EVKI does not bind to the PDZ domains of GRIP; consequently, it did not interfere with GluR2–GRIP interaction (**Fig. 1b**). However, the EVKI

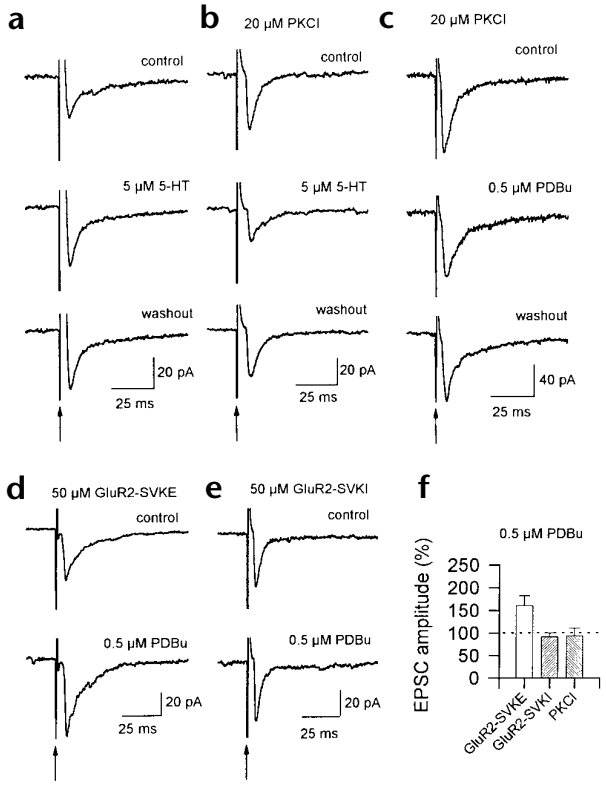

Fig. 4. Postsynaptic PKC activation is necessary and sufficient for synaptic facilitation by 5-HT. (**a**) 5 μM 5-HT induces a persistent potentiation of EPSC amplitude in representative traces from a dorsal horn neuron. The three traces illustrate EPSCs before drug application (control), at the end of a 10-min bath application of 5 μM 5-HT and 30 min after return to control bath solution (washout). (**b**, **c**) Loading postsynaptic neurons with the PKC inhibitor PKCI (20 μM) abolished the facilitatory effect of both 5-HT (5 μM; **b**) and the PKC activator PDBu (0.5 μM; **c**) on EPSCs in experiments conducted as in (**a**). Application through the patch pipet of 50 μM GluR2-SVKI (**e**), but not 50 μM GluR2-SVKE (**d**) prevented facilitation of dorsal horn synaptic transmission by bath application of 0.5 μM PDBu. (**f**) Pooled data shows that 50 μM GluR2-SVKI (n = 6) and 20 μM PKCI (n = 5), but not 50 μM GluR2-SVKE (n = 7), blocked the potentiation of EPSCs by 0.5 μM PDBu.

mutant can still bind to the PDZ domain of PICK1 (R. Huganir, personal communication; see also ref. 10). GluR2-EVKI (50 μM) had no effect on 5-HT-induced synaptic facilitation ($n = 4$; **Fig. 3d**), suggesting that the GluR2–PICK1 interaction is not critical for this form of synaptic modulation. Rather, this finding suggests a requirement of interaction between GluR2 and GRIP/ABP for 5-HT-induced sensory synaptic facilitation. The *C* terminus of PKCα also binds to the PDZ domain of PICK1[24]. However, GluR2-EVKI's inability to inhibit synaptic facilitation argues against the notion that inhibition by GluR2-SVKI involves displacement of PKC from PICK1 and mislocalization of PKC in the cell.

A subset of central excitatory synapses show no fast AMPA receptor-mediated EPSCs at –70 mV but do reveal a slow response mediated by NMDA receptors at +40 mV, when their voltage-dependent Mg^{2+} blockade is relieved[3,25–29]. These so-called 'silent' synapses are found between primary sensory afferents and spinal dorsal horn neurons[14,30] and are revealed by lowering the intensity of DREZ stimulation until fast synaptic transmission at –70 mV ceases. By applying low doses of 5-HT, silent synapses can be converted to functional synapses that exhibit fast AMPA receptor-mediated EPSCs[14]. To determine whether PKC mediates this conversion, we tested the ability of 0.5 μM PDBu to induce fast EPSCs in dorsal horn neurons during low-intensity stimulation (holding potential, –70 mV). (In all of the following experiments, an NMDA receptor-mediated current could be evoked in baseline conditions by switching the holding potential to +40 mV.) Indeed, in five experiments where the recording electrode was loaded with 50 μM GluR2-SVKE, bath application of the PKC activator consistently revealed fast excitatory responses in previously silent pathways (**Fig. 5b**), an effect which was rapid (within 5 min) and persistent (lasting > 30 min; **Fig. 5b**). The rapid onset of this phenomenon supports the notion that activation of silent synapses by PKC involves the recruitment of existing, rather than newly synthesized, AMPA receptors. Protein–protein interactions involving the GluR2/3 *C* terminus are critical for the activation of silent synapses by 5-HT or PDBu: when the recording electrode was filled with 50 μM GluR2-SVKI, application of 5 μM 5-HT ($n = 6$; **Fig. 5a**) or 0.5 μM PDBu ($n =$ 5 out of 6 experiments; **Fig. 5b** and **c**) failed to induce fast EPSCs except in one experiment, where PDBu application evoked small, fast, synaptic responses. In contrast, NMDA currents, recorded at a holding potential of +40 mV, were similarly decreased with either GluR2-SVKI ($n = 6$; 14.9 ± 5.5% of control) or no peptide ($n = 8$, 18.7 ± 4.3%) in the recording pipet. In addition to an enhancement of short-latency, monosynaptic responses, PDBu also induced some late synaptic responses not characterized here. Unlike monosynaptic responses, these late synaptic responses were not affected by GluR2-SVKI, suggesting that they may reflect presynaptic effects or effects of PKC on interneurons.

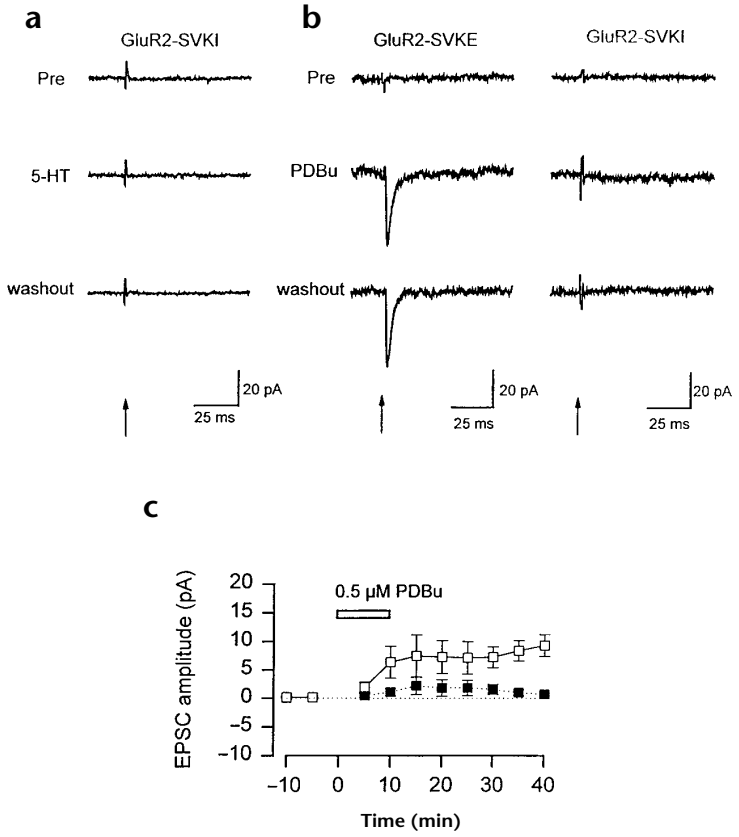

Fig. 5. GluR2-SVKI inhibits the activation of silent glutamatergic synapses. (**a**) 5 μM 5-HT activates silent synapses in dorsal horn neurons[14]. Representative traces show that this activation was prevented when 50 μM GluR2-SVKI was present in the patch pipet. Traces are shown at time points before 5-HT exposure (Pre), at the end of a 10-min bath application of 5 μM 5-HT, and 30 min after return to control bath solution (washout). (**b**, **c**) Representative traces (**b**) and pooled data (**c**) show that silent synapses in dorsal horn neurons were activated by 0.5 μM PDBu in the presence of 50 μM GluR2-SVKE ($n = 5$) or no peptide ($n = 5$; data were not different between these two control conditions and were pooled; open squares). However, PDBu failed to activate silent synapses in most neurons loaded with 50 μM GluR2-SVKI ($n = 6$; see text; closed squares). Traces are shown at time points before PDBu exposure (Pre), at the end of a 10-min application of 0.5 μM PDBu and 30 min after return to control bath solution (washout).

Discussion

We provide direct physiological evidence that PKC and interactions of the GluR2/3 *C* terminus are important for the 'unsilencing' of glutamatergic synapses between sensory afferents and spinal cord dorsal horn neurons. We favor a mechanism for 5-HT-induced synaptic facilitation in which recruitment of AMPA receptors at the postsynaptic membrane involves binding of GluR2/3 cytoplasmic tails to postsynaptic PDZ proteins such as GRIP/ABP. The possibility cannot be excluded that the GluR2/3 *C* terminus may be required for AMPA-receptor activation by post-translational modification; for instance, the *C* terminus itself may be a site of phosphorylation, or it may form a complex with a protein kinase with AMPA receptors. However, we think it unlikely that the GluR2-SVKI peptide acts as a substrate and competitive inhibitor of PKC, since the control peptide GluR2-SVAI still inhibited synaptic facilitation like the wild-type GluR2 *C* terminus. There was an absolute correlation between the ability of the various peptides to interfere with the GluR2-GRIP interaction and their ability to prevent 5-HT-induced synaptic facilitation. The GluR2-SVKI and -SVAI peptides (which inhibit facilitation) most likely target a PDZ protein that binds specifically to GluR2/3. Recognition of a short *C*-terminal peptide sequence with strong dependence on the last residue but weak preference at the –1

residue (compare the properties of SVKE and SVAI peptides) is typical for PDZ domains in general, and true specifically for the PDZ binding partners of the GluR2/3 *C* terminus.

Our findings implicate PDZ binding directly in a mechanism of synaptic plasticity and outline a role for AMPA receptor–PDZ interactions in the activation of silent synapses. Moreover, we show that PKC activation is a critical intermediary step linking 5-HT receptor stimulation and synaptic facilitation in the dorsal horn. The molecular mechanisms by which PKC activates silent synapses, and how this activation involves GluR2/3–PDZ interactions, remain unclear.

Although loading neurons with a GluR2/3 *C*-terminal peptide abolished synaptic facilitation, it had no effect on baseline synaptic responses or responses to applied glutamate. Several possibilities could account for this specificity. In the basal state, the interaction between synaptic AMPA receptors and partner PDZ proteins may be stable, and hence, resistant to competition by an interfering peptide, whereas during synaptic enhancement, recruitment of AMPA receptors requires formation of new PDZ-mediated associations that are sensitive to competitive inhibition. Alternatively, basal AMPA receptor responses may involve receptors that are not interacting with PDZ-domain proteins at all; that is, functional AMPA receptors already resident at the postsynaptic membrane are not bound to PDZ proteins, whereas newly recruited AMPA receptors may need to interact with PDZ proteins during a critical step.

The existence of ineffective, or silent, sensory synapses in the spinal cord dorsal horn was first proposed by Wall[31]. However, it is unclear whether ineffective sensory transmission might reflect the failure of a reduced postsynaptic current to depolarize a neuron to action potential threshold or, rather, an increased threshold in a neuron with normal synaptic responses. Ineffective dorsal horn synapses lack fast postsynaptic responses and can be rendered functional through the recruitment of AMPA receptors[14]. Various studies demonstrate that the membrane expression of AMPA receptors in spinal cord neurons can indeed be modulated. Activity-dependent changes in synaptic AMPA-receptor accumulation are reported in the spinal cord[32], as are changes in the quantity of postsynaptic AMPA receptors[33,34] and the morphology of dendritic structures[35] after tissue or nerve injury. Our findings provide further support for the idea that modulation of glutamate-receptor distribution in postsynaptic membranes is critical for the regulation of synaptic efficacy.

Vesicle-mediated membrane insertion of AMPA receptors may contribute to synaptic plasticity in the hippocampus, where silent synapses are thought to play a role in long-term potentiation (LTP)[3,25–27]. Postsynaptic, NSF-dependent membrane fusion events are necessary for LTP[36]. Moreover, NSF, a well-characterized protein involved in vesicular transport binds to the membrane-proximal part of the GluR2 cytoplasmic tail[37–40]. Peptides that inhibit NSF-GluR2 binding not only prevent LTP[36], but, in contrast to the action of GluR2-SVKI, also cause a rundown of basal AMPA receptor-mediated currents[37,39] (but see ref. 36). Our findings with the GluR2-SVKI peptide indicate, therefore, that two distinct regions of the GluR2 cytoplasmic tail are critical for potentiation of AMPA receptor responses: the membrane-proximal site that interacts with NSF and the *C*-terminal motif that binds to PDZ proteins. The proximity of these domains in the GluR2 protein and their related functions suggest possible functional interaction between NSF and the PDZ-containing binding partners of GluR2. How these different GluR2-binding proteins cooperate with each other to recruit AMPA receptors to silent synapses promises to be a fruitful area of future research.

A potential pathological consequence of silent-synapse activation in the spinal cord is enhancement of sensory transmission during chronic pain[14,31]. AMPA receptors are expressed in dorsal horn neurons at synapses receiving either high- or low-threshold inputs[41,42]. Activation of silent synapses with low-threshold afferents might cause postsynaptic cells to increase firing rate in response to non-noxious stimuli. Indeed, increases in postsynaptic AMPA receptor density and activation of silent synapses could alter various electrophysiological properties of dorsal horn sensory neurons, including increased receptive fields, enhanced responses to noxious and non-noxious stimuli, decreased firing threshold and increased background activity[43–45]. In this way, silent synapse activation could underlie two features of chronic pain, hyperalgesia (where the intensity of responses to noxious stimuli is increased over baseline) and allodynia (where the nociceptive threshold is decreased and a normally non-noxious stimulus, such a gentle touch, can induce pain). Further study of the processes regulating GluR2/3 *C*-terminal interactions, the trafficking and membrane insertion of AMPA receptors and PKC-mediated intracellular signaling pathways could suggest targets for the therapeutic control of chronic pain.

Methods

Electrophysiology in spinal cord slices. Spinal cord slices from P4–21 rats were prepared as described[14]. Whole-cell recordings were made using 5–10 MΩ electrodes without fire polishing and filled with a solution containing 110 mM Cs-$MeSO_3$, 5 mM $MgCl_2$, 1 mM EGTA, 40 mM HEPES sodium, 2 mM MgATP and 0.1 mM Na_3GTP at pH 7.2. The osmolarity was adjusted to 295–300 mOsm. Membrane potential was clamped at –70 mV (liquid junction potential not corrected). Series resistance monitored throughout experiments was 15–40 MΩ; currents were filtered at 1 kHz and digitized at 5 kHz. Postsynaptic EPSCs were evoked at 0.05–0.02 Hz with a bipolar tungsten electrode placed near the central end of the dorsal root or DREZ, producing a stimulus width of 0.1–0.4 ms. Monosynaptic EPSCs were identified using two criteria. First, the response latency did not change with increasing intensities of electrical stimulation, and second, following high-frequency stimulation (50 Hz), EPSC amplitude was reduced but response latency was unchanged[14]. Only monosynaptic EPSCs were used in the present study. Bicuculline methiodide (10 μM) and strychnine hydrochloride (1 μM) were added to the perfusion solution. Statistical comparisons were made with the use of one-way analyses of variance (ANOVAs: Dunnett test for *post-hoc* comparison) or Student's *t*-test. $p < 0.05$ was considered significant. Error bars represent standard error of the mean.

Electrophysiology in cultured neurons. Neurons were dissociated from the dorsal half of spinal cord slices and maintained for 7 to 14 days in culture using standard methods[7]. Pipets were filled with 140 mM Cs-$MeSO_3$, 10 mM EGTA, 10 mM HEPES, 5 mM CsCl, 5 mM $MgCl_2$, 5 mM ATP and 1 mM GTP at pH 7.4. Drugs were dissolved in 160 mM NaCl, 10 mM HEPES and 2 mM $CaCl_2$ plus 500 nM tetrodotoxin and 2 μM MK-801 at pH 7.4 and applied by rapid, local perfusion from a multibarreled pipet.

Western blotting. Spinal cord dorsal horn and cerebral cortex were dissected from adult rats and homogenized in 25 mM Tris-HCl, pH 7.4, with 5mM EDTA, 5mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg per ml of aprotinin, 10 μg per ml of leupeptin and 10 μg per ml pepstatin. 10 μg protein from these regions were separated by SDS-PAGE and immunoblotted using antibodies specific for GRIP[46] and GluR2/3 (PharMingen, San Diego, California).

GST-pulldown assays. COS-7 cells were transfected with GluR2 cDNA in the mammalian expression vector pGW1-CMV using Lipofectamine (GIBCO-BRL, Life Technologies, Grand Island, New York). Two days after transfection the cells were extracted in RIPA buffer and 100 μl of the extract was incubated with glutathione sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, New Jersey; bed volume ≈ 8 μl)

coupled to 6 μg GST or GST-GRIP fusion protein containing PDZ4–6 of GRIP[46] for 2 h at 4°C. Bound GluR2 was eluted with SDS sample buffer and immunoblotted with GluR2/3 antibodies (PharMingen, San Diego, California) and HRP-conjugated secondary antibodies and visualized with ECL chemiluminescence (Amersham, Arlington Heights, Illinois). GluR2 *C*-terminal peptides (100 μM) were preincubated with the coupled glutathione sepharose beads for 1 hour before adding the cell lysate.

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