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Dorsal Horn α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Trafficking in Inflammatory Pain

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ABSTRACT

Activation of synaptic *N*-methyl-D-aspartic acid receptor and its intracellular downstream signals in dorsal horn neurons of spinal cord contribute to central sensitization, a mechanism that underlies the development and maintenance of pain hypersensitivity in persistent pain. However, the molecular process of this event is not understood completely. Recently, new studies suggest that peripheral inflammatory insults drive changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit trafficking *via N*-methyl-D-aspartic acid receptor-triggered activation of protein kinases in dorsal horn and raise the possibility that such changes might contribute to central sensitization in persistent pain. This review presents current evidence regarding the changes that occur in the trafficking of dorsal horn α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunits GluR1 and GluR2 under persistent inflammatory pain conditions and discusses the potential mechanisms by which such changes participate in the development and maintenance of inflammatory pain.

INFLAMMATION and tissue or nerve injury cause persistent, or chronic, pain, which is characterized by an enhanced response to noxious stimuli (hyperalgesia) and pain in response to normally innocuous stimuli (allodynia). In dorsal horn of the spinal cord, a specific form of synaptic plasticity known as central sensitization is believed to be a mechanism that underlies the development and maintenance of hyperalgesia and allodynia in persistent pain.[1,2] An understanding at the molecular level of how central sensitization is induced and maintained in dorsal horn neurons could lead to the development of novel therapeutic targets.

Central sensitization in dorsal horn can be modulated by altering the presynaptic release of neurotransmitters (*e.g.*, glutamate and substance P) from the primary afferent terminals and/or by altering the number, types, and properties of postsynaptic membrane receptors (*e.g.*, glutamate receptors) on postsynaptic membranes of dorsal horn neurons.[1,2] The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is a glutamate ionotropic receptor that is integral to plasticity and excitatory synaptic transmission at synapses in the central nervous system.[3,4] Studies from *in vitro* and *in vivo* systems have revealed that changes in postsynaptic membrane trafficking or in synaptic targeting of AMPARs alter excitatory synaptic strength. These changes might contribute to central mechanisms that underlie physiologic and pathologic processes, such as synaptic plasticity, neurotoxicity, and stroke.[5–8] Recent studies have shown that peripheral inflammatory insults drive changes in synaptic AMPAR trafficking in dorsal horn[9–12] that might contribute to dorsal horn central sensitization in persistent inflammatory pain.

This review presents current evidence regarding changes that occur in the trafficking of AMPAR subunits GluR1 and GluR2 in dorsal horn neurons after peripheral inflammation and discusses potential mechanisms by which such changes participate in the development and maintenance of inflammatory pain. The role of synaptic AMPAR trafficking in relation to other important mechanisms of activity-dependent sensitization (*e.g.*, Ca^{2+} influx) will also be discussed.

AMPAR Subunits and Their Expression in Dorsal Horn

In the central nervous system, AMPARs are assembled from four subunits, GluR1–4.[3,4] These subunits are composed of approximately 900 amino acids and share 68–74% amino acid sequence identity.[13] Each subunit comprises an *N*-ter-

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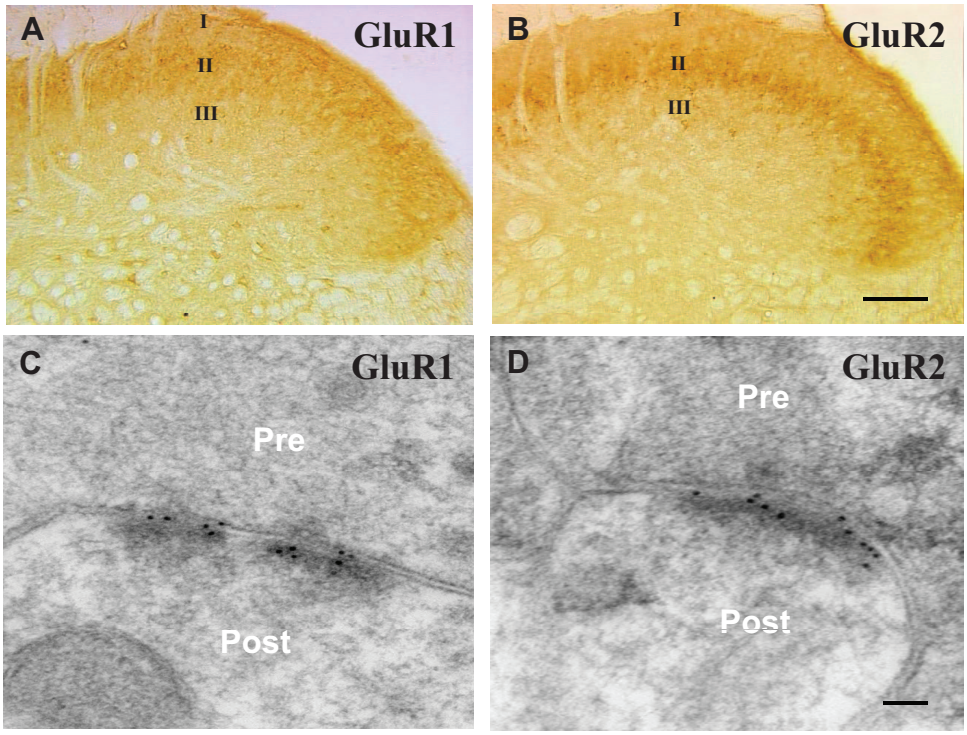

Fig. 1. Expression and distribution of synaptic GluR1 and GluR2 in dorsal horn of naïve rats. (*A*, *B*) Representative photographs showing that GluR1 immunoreactivity is distributed mainly in laminae I and II and that GluR2 immunoreactivity is predominantly in lamina II. Scale bar: 200 μm. (*C*, *D*) Examples of postsynaptic immunogold labeling for GluR1 and GluR2 in superficial dorsal horn synapses illustrate labeling in the synapses. Post = postsynaptic structure; Pre = presynaptic terminals. Scale bar: 100 nm.

minal extracellular segment, a ligand-binding domain, a receptor-channel domain, and an intracellular *C*-terminal domain.[14] In the ligand-binding domain, two polypeptide segments represent the agonist-recognition sites. This domain also functionally interacts with stargazin, an auxiliary subunit of AMPARs.[15] The receptor channel domain consists of four hydrophobic segments (M1–M4). M1, M3, and M4 cross the membrane, whereas M2 faces the cytoplasm as a reentered loop that forms part of the channel pore.[16,17] Thus, among receptor channel domains, M2 controls the flow of ions (including Ca^{2+}) through the AMPAR channel. The *C*-terminal intracellular domain includes multiple protein phosphorylation sites for various known protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and protein kinase A, and several binding sites (or motifs) for various other proteins, such as the scaffolding proteins (*e.g.*, postsynaptic density protein 95) that contain a specific peptidergic domain called PDZ, which is named for the proteins in which the sequence was first identified (Postsynaptic density protein 95/Discs large/Zonula occludens 1).[14,17]

Functional AMPARs are homomeric or heteromeric tetramers of GluR subunits. Homomeric channels formed from GluR1, GluR3, or GluR4 are Ca^{2+} permeable and inwardly rectifying (that is, the channel passes current [positive charge] more easily in the inward direction [into the cell]). Homomeric GluR2 channels, however, express poorly on their own and lack Ca^{2+} permeability and inward rectification, because the GluR2 subunit contains a positively charged arginine at a critical position in the pore-forming M2 segment.[4] Incorporation of GluR2 into heteromeric AMPARs strongly reduces Ca^{2+} permeability and modifies current rectification and macroscopic channel conductance.[3]

Although all four AMPAR subunits are found within the spinal dorsal horn, GluR1 and GluR2 are the most abundant and are highly concentrated on the postsynaptic neuronal membranes in the superficial dorsal horn (fig. 1).[18,19] Thus, under normal conditions, dorsal horn neurons may express one type or a mixture of Ca^{2+}-permeable and Ca^{2+}-impermeable AMPARs.[20,21]

Noxious Insults Upset the Balance of AMPAR Subunit Recycling in Dorsal Horn

AMPAR subunits are synthesized and assembled in the rough endoplasmic reticulum and golgi of neuronal cell bodies and then inserted into the plasma membrane at the soma. Receptors inserted in the soma may travel to extrasynaptic sites *via* lateral diffusion.[16,21] The subunits can also be synthesized locally in dendrites. Subunit messenger RNA is trafficked out into dendrites *via* an RNA–protein complex that travels along the cytoskeleton. Messenger RNA can be translated by local polyribosomes in response to neuronal activity.[16,17] Proteins translated in the dendrites are processed *via* dendrite golgi outposts and travel to extrasynaptic sites. Extrasynaptic receptors diffuse laterally into the synapse,

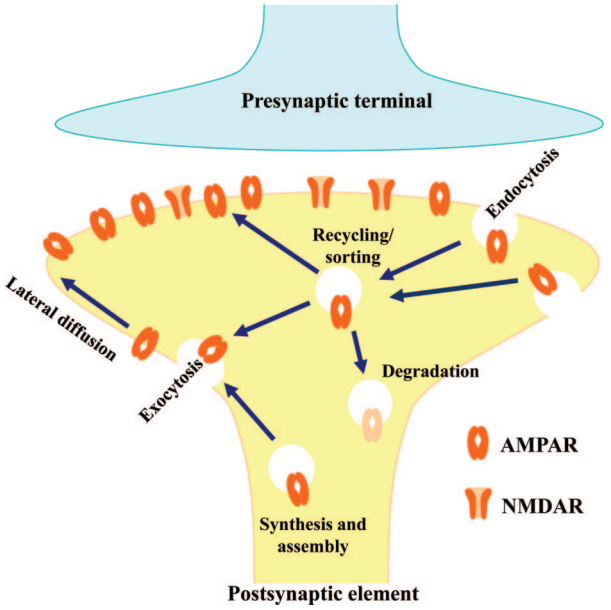

Fig. 2. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking at a neuronal synapse. After synthesis and assembly in the neuronal cell body and local dendrites, AMPAR subunits are transported to extrasynaptic membrane *via* exocytosis and then diffuse laterally into the synaptic membrane. At the postsynaptic membrane, AMPAR subunits can be recycled back into the intracellular space *via* endocytosis. Some of the endocytosed subunits are degraded, whereas the remaining subunits are recycled back to the synaptic membrane and/or to extrasynaptic membrane *via* exocytosis. Thus, the number of AMPAR subunits expressed on the synaptic membrane is dependent on the balance between these processes. NMDAR = *N*-methyl-D-aspartic acid receptor.

where they are trapped by scaffolding proteins (*e.g.*, postsynaptic density protein 95).[17] Synaptic AMPAR subunits can be recycled back to the intracellular compartment *via* clathrin-mediated internalization (endocytosis) (fig. 2).[17,22] The endocytosed receptors in the endosome are then either recycled back to the plasma membrane *via* exocytosis or targeted to the lysosome for degradation (fig. 2).[17,22] The number of subunits expressed on the synaptic membrane is dependent on the balance between these processes.

By using biochemical and morphologic approaches, researchers have shown that peripheral noxious insults upset the balance of AMPAR subunit recycling between the membrane and cytosol in the dorsal horn. In one study, capsaicin-induced acute inflammation in the colon rapidly and significantly increased the amount of membrane GluR1 protein and correspondingly decreased the level of cytosolic GluR1 in dorsal horn neurons, without affecting total GluR1 or GluR2 protein expression (table 1).[9] In another study, electron microscopy revealed that capsaicin injection into a rat hind paw increased the density of GluR1-containing AMPARs and the ratio of GluR1 to GluR2/3 in postsynaptic membranes contacted by noxious primary afferent terminals that lack substance P.[23] In addition, the injection of formalin (an inflammatory agent) into the intraplantar region of a hind paw produced an increase in the level of GluR1 in the plasma membrane of dorsal horn neurons (table 1).[24]

Interestingly, injection of complete Freund's adjuvant (CFA) into a hind paw, which produces long-lasting peripheral inflammation and persistent inflammatory pain, led to changes in subcellular localization of both GluR1 and GluR2 in dorsal horn neurons but did not alter their total expression and distribution in dorsal horn.[11,12] The amount of cytosolic GluR2 was markedly increased, and the amount of membrane-bound GluR2 was significantly decreased in rat dorsal horn during the maintenance phase of CFA-induced pain hypersensitivity (at least 3 days postinjection) (table 1).[11,12] Conversely, the level of GluR1 was significantly decreased in cytosol and increased in the neuronal membrane 1 day post-CFA (table 1).[11,12] Furthermore, we and others observed that the level of synaptic GluR2 was reduced in superficial dorsal horn (laminae I–II) neurons 1 day post-CFA injection.[10,12] However, none of these studies showed any changes in either GluR1 or GluR2 during the development phase (2 h post-CFA).[10,12] This evidence implies that acute (*e.g.*, capsaicin) and persistent (*e.g.*, CFA) inflammatory insults may induce different changes in dorsal horn AMPAR subunit trafficking.

Potential Mechanisms of AMPAR Trafficking Regulation in Inflammatory Pain

The molecular mechanisms by which peripheral noxious insults alter dorsal horn AMPAR subunit trafficking are unclear, but they might be associated with nociceptive information-induced phosphorylation of the serine (Ser) residues of both GluR1 and GluR2 *C*-termini in the spinal cord. Three major phosphorylation sites on the Ser residues of the GluR1 *C*-terminus have been identified: Ser^{845}, Ser^{831}, and Ser^{818}. Ser^{845} is specifically phosphorylated by protein kinase A,[25] Ser^{831} by PKC and CaMKII,[25,26] and Ser^{818} by PKC.[27] The change in phosphorylation status of these three sites directly

Table 1. GluR1 and GluR2 Trafficking in Dorsal Horn after Inflammation

	GluR1			GluR2		
Model	Cytosol	Membrane	Total	Cytosol	Membrane	Total
Capsaicin	↓	↑	—	—	—	—
Formalin	↓/↑	↑	N/A	N/A	N/A	N/A
CFA	↑	↑	—	↑	↓	—

CFA = complete Freund's adjuvant; N/A = not available; ↓ = decrease; ↑ = increase; ↓/↑ = decrease at early and increase at late; — = no change.

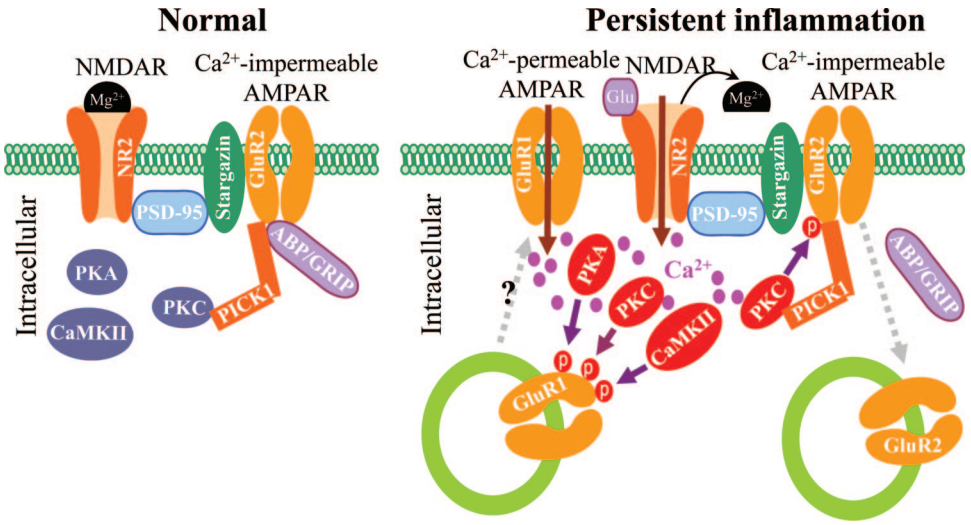

Fig. 3. Trafficking of dorsal horn synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunits GluR1 and GluR2 in inflammatory pain. AMPARs are physically coupled to *N*-methyl-D-aspartic acid receptors (NMDARs) by a PSD-95-stargazin linkage with GluR1/2/4 and NR2A/2B and to PKC by PICK1 with GluR2 and PKC. Under normal conditions, Mg^{2+} blocks NMDAR activity, and intracellular kinases are inactive. GluR2 is stabilized at the synaptic membrane by its anchoring protein, ABP/GRIP. Peripheral inflammatory insult triggers the removal of Mg^{2+} and NMDAR activation. Ca^{2+} influx through NMDARs activates intracellular Ca^{2+}-dependent kinases, including PKC, PKA, and CaMKII. These activated kinases phosphorylate GluR1 and promote GluR1 membrane insertion. The activated PKC also phosphorylates GluR2 at Ser.[880] This phosphorylation disrupts the binding of GluR2 to ABP/GRIP and promotes GluR2 internalization. GluR1 membrane insertion and GluR2 internalization lead to a switch from Ca^{2+}-impermeable AMPARs (GluR2-containing AMPARs) to Ca^{2+}-permeable AMPARs (GluR2-lacking AMPARs). The increase in Ca^{2+}-permeable AMPARs further enhances Ca^{2+} influx and the activity of intracellular Ca^{2+}-dependent kinases, including the three kinases described. This positive feedback loop might underlie the mechanism of central sensitization in the maintenance of inflammatory pain. ABP = AMPAR-binding protein; CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II; Glu = glutamate; GRIP = glutamate receptor-interacting protein; NMDAR = *N*-methyl-D-aspartic acid receptor; NR2 = *N*-methyl-D-aspartic acid receptor subunit 2; PICK1 = protein interacting with C kinase 1; PKA = protein kinase A; PKC = protein kinase C; PSD-95 = postsynaptic density protein 95.

impacts electrophysiologic properties, subunit composition, and/or expression of synaptic AMPARs. Protein kinase A phosphorylation of GluR1 at Ser^{845} increases the channel open probability of AMPARs and the peak amplitude of their currents, stabilizes GluR1 in the plasma membrane, enhances the incorporation of GluR1-containing AMPARs into the synaptic membrane, and decreases AMPAR internalization.[28–30] Phosphorylation of GluR1 on Ser^{831} (the PKC/CaMKII site) increases the signal channel conductance of synaptic AMPARs,[31] but it does not seem to influence membrane insertion. Rather, CaMKII activation drives GluR1-containing AMPARs to synapses by a mechanism that requires the binding of GluR1 to its scaffolding protein.[32] In contrast, phosphorylation of GluR1 Ser^{818} by PKC might be involved in promoting synaptic GluR1 membrane insertion, as preventing Ser^{818} phosphorylation blocks PKC-driven synaptic incorporation of GluR1.[27] However, how these phosphorylation sites promote GluR1 membrane insertion is still unknown.

Capsaicin or CFA injection increases phosphorylation at Ser^{845} and Ser^{831} of GluR1 in dorsal horn neurons.[33–35] Phosphorylation at these serine residues might be involved in dorsal horn GluR1 membrane insertion in response to acute (capsaicin) or persistent (CFA) inflammatory insults. To confirm this conclusion, it will be very interesting to investigate how targeted mutation of Ser^{845}, Ser^{831}, and Ser^{818} affects inflammatory insult-induced changes in dorsal horn neuronal synaptic GluR1 trafficking and inflammation-induced pain hypersensitivity.

GluR2 contains a PDZ-binding motif at its *C*-terminus that interacts with the PDZ domains of two postsynaptic scaffolding proteins, protein interacting with C kinase 1 (PICK1) and AMPAR-binding protein (ABP)/glutamate receptor-interacting protein (GRIP). ABP/GRIP anchors and stabilizes GluR2 at the synaptic membrane,[36–38] whereas PICK1 recruits PKC to ABP/GRIP-GluR2 complexes, leading to phosphorylation of GluR2 at Ser^{880} (fig. 3).[39–41] Evidence from cultured brain neurons and human embryonic kidney 293 cells showed that GluR2 phosphorylation at Ser^{880} by PKC disrupted GluR2 binding to ABP/GRIP, but not binding to PICK1, and promoted GluR2 internalization.[39,40,42,43]

Our recent evidence indicates that *N*-methyl-D-aspartic acid receptor (NMDAR)-triggered PKC phosphorylation at GluR2 Ser^{880} is responsible for GluR2 internalization in dorsal horn neurons after persistent inflammation. In dorsal horn of normal rat, GluR2 binds to both GRIP1 and PICK1, whereas PICK1 also interacts with PKCα (fig. 3).[12] NMDAR and PKC activation significantly increased the level of GluR2 phosphorylation at Ser^{880} and reduced the surface expression of GluR2 in cultured dorsal horn neurons.[12] In contrast, the activation of AMPARs or group 1 metabotropic glutamate receptors had no effect.[12] In an *in vivo* study, CFA injection into the hind paw of mice and rats

induced the GluR2 phosphorylation at Ser^{880}, reduced the binding affinity of GluR2 to GRIP1, and promoted the Glu2 internalization in dorsal horn neurons during the maintenance phase.[12] These effects were attenuated by an intrathecally administered NMDAR antagonist or PKC inhibitor.[12] More importantly, targeted mutation of GluR2 phosphorylation site Ser^{880} attenuated CFA-induced GluR2 internalization in dorsal horn neurons.[12] These findings suggest that dorsal horn NMDARs are activated and that Ca^{2+} could flow inward through NMDAR channels during peripheral inflammation (fig. 3). The increase in intracellular Ca^{2+} would promptly activate PKC to phosphorylate GluR2 at Ser^{880}; this phosphorylation would disrupt GluR2 binding to ABP/GRIP and promote GluR2 internalization at postsynaptic dorsal horn neurons (fig. 3). This conclusion is strongly supported by evidence that NMDAR-triggered GluR2 internalization *in vitro* requires Ca^{2+} influx directly through NMDARs.[44,45] In addition, there is a physical link between AMPARs and NMDARs or PKC at synapses in dorsal horn of the spinal cord (fig. 3).[12] Postsynaptic density protein 95 binds to both NMDAR subunits NR2A/2B and stargazin, whereas stargazin interacts with GluR1/2/4. It seems that AMPARs are physically coupled to the NMDAR complex by a postsynaptic density protein 95-stargazin linkage. PICK1 recruits PKCα to GluR2 in dorsal horn neurons.[12] Thus, this physical coupling provides a molecular basis by which Ca^{2+} can enter the cell through the NMDAR channel and promptly activate intracellular protein kinases (*e.g.*, PKC) that can then phosphorylate GluR1 and GluR2 in dorsal horn neurons (fig. 3). Taken together, the evidence clearly supports the premise that GluR2 phosphorylation at Ser^{880} triggered by NMDAR/PKC activation contributes to CFA-induced dorsal horn GluR2 internalization during the maintenance phase.

In addition to PICK1 and ABP/GRIP, other proteins that interact with the GluR2 *C*-terminal may also be involved in changes of synaptic GluR2 trafficking in dorsal horn neurons in inflammatory pain. For example, *N*-ethylmaleimide-sensitive fusion protein binds to GluR2 and helps to maintain the synaptic expression of GluR2-containing AMPARs. *N*-ethylmaleimide-sensitive fusion protein also displaces PICK1 from the PICK1-GluR2 complex and thereby facilitates the delivery to or stabilization of GluR2 at the plasma membrane. CFA injection has been shown to reduce *N*-ethylmaleimide-sensitive fusion protein expression in the spinal cord.[10] This reduction might also contribute to CFA-induced dorsal horn GluR2 internalization.

AMPAR Trafficking and Central Sensitization in Inflammatory Pain

Based on the evidence presented thus far, inflammation-induced changes in AMPAR subunit trafficking at postsynaptic membranes of dorsal horn neurons might be involved in the maintenance of central sensitization in persistent inflammatory pain (fig. 3). CFA-induced time-dependent changes in GluR2 internalization and GluR1 membrane insertion in dorsal horn neurons correlate with the time course of changes in CFA-induced pain hypersensitivity during the maintenance period,[11,12] indicating that these changes may be markers for pain hypersensitivity during the maintenance of persistent inflammatory pain. We have reported that activation of the spinal cord NMDARs and PKC induces dorsal horn GluR2 internalization during the maintenance period of CFA-induced inflammatory pain.[12] Given that the spinal cord NMDARs and PKC are critical for the maintenance of chronic inflammatory pain,[46] dorsal horn GluR2 internalization might underlie the mechanism by which activation of NMDARs and PKC maintains inflammatory pain (fig. 3). Indeed, preventing dorsal horn GluR2 internalization through targeted mutation of the GluR2 PKC phosphorylation site impairs CFA-evoked mechanical allodynia and thermal hyperalgesia during the maintenance period.[12] Moreover, CFA-induced GluR2 internalization and GluR1 membrane insertion lead to an increase in AMPAR Ca^{2+} permeability in dorsal horn neurons (fig. 3). This phenomenon was illustrated by electrophysiologic patch-clamp recordings of rat dorsal horn neurons. This experiment showed that the number of Ca^{2+}-permeable AMPARs in the superficial dorsal horn was significantly increased during the maintenance phase of CFA-induced persistent inflammatory pain.[10,12,47] The increase in intracellular Ca^{2+} concentration should initiate or potentate a variety of Ca^{2+}-dependent intracellular cascades that are associated with the mechanisms of pain hypersensitivity during inflammatory pain states. Thus, GluR2 internalization and GluR1 membrane insertion in dorsal horn may participate in the maintenance of CFA-induced pain hypersensitivity by increasing synaptic AMPAR Ca^{2+} permeability. The latter leads to more increases in intracellular Ca^{2+} concentration, which further enhances activation of Ca^{2+}-dependent intracellular signals (*e.g.*, PKC, CaMKII, and protein kinase A activation) in dorsal horn. This positive feedback that occurs through alteration of AMPAR subunit trafficking and increases in Ca^{2+}-permeable AMPARs in dorsal horn neurons could be crucial for the maintenance of pain hypersensitivity in persistent inflammatory pain (fig. 3).

In summary, new research has brought to light distinct changes that occur in dorsal horn AMPAR subunit trafficking in acute and persistent inflammatory pain. Our recent work showed that peripheral noxious input induced by incision pain did not produce a significant change in dorsal horn AMPAR subunit trafficking (data not shown). These findings suggest that different peripheral nociceptive insults may induce distinct changes in spinal cord AMPAR subunit trafficking. It will be very important to further characterize AMPAR subunit trafficking in dorsal horn under other persistent pain conditions (such as neuropathic pain) and to investigate other potential molecular mechanisms to fully understand the role of spinal cord AMPAR trafficking in the central sensitization that underlies persistent pain. Such studies will not only provide new insight into the central

mechanisms of persistent pain but might also point the way to a new strategy for the prevention and treatment of persistent pain.

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