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Activity-dependent Synaptic Recruitment of Neuroligin 1 in Spinal Dorsal Horn Contributed to Inflammatory Pain

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Abstract—Neuroligin 1 (NLGN1), a cell adhesion molecule present at excitatory glutamatergic synapses, has been shown to be critical for synaptic specialization and N-methyl-D-aspartate (NMDA)-subtype glutamate receptor-dependent synaptic plasticity. Whether and how NLGN1 is engaged in nociceptive behavioral sensitization remains largely unknown. In this study, we found an activity-dependent regulation of NLGN1 synaptic expression in pain-related spinal cord dorsal horns of mice. The enhancement of neuronal activity by pharmacological activation of NMDA receptor (NMDAR) or removal of GABAergic inhibition in intact mice significantly increased NLGN1 concentration at synaptosomal membrane fraction. Intraplantar injection of complete Freund's adjuvant (CFA) also increased the NLGN1 expression at synapses. NMDAR might act through Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Src-family protein tyrosine kinase member Fyn to induce the synaptic redistribution of NLGN1. We also found that one of the important roles of NLGN1 was to facilitate the clustering of NMDAR at synapses. The NLGN1-targeting siRNA suppressed the synaptic expression of GluN2B-containing NMDAR in CFA-injected mice and meanwhile, attenuated the inflammatory mechanical allodynia and thermal hypersensitivity. These data suggested that tissue injury-induced synaptic redistribution of NLGN1 was involved in the development of pain hypersensitivity through facilitating the synaptic incorporation of NMDARs.

Key words: neuroligin, NMDA receptor, CaMKII, Fyn, pain hypersensitivity.

INTRODUCTION

Neuroligins (NLGNs) are a family of postsynaptic adhesion molecules that are critical for the differentiation of excitatory and inhibitory synapses (Goda and Davis, 2003; Sudhof, 2008; Bemben et al., 2015). Among the four identified neuroligin isoforms (NLGN1–NLGN4), Neuroligin 1 (NLGN1) is preferentially localized at glutamatergic excitatory synapses and interacts with the postsynaptic scaffolding protein PSD-95 via a specific PDZ-binding motif (Irie et al., 1997; Song et al., 1999; Levinson et al., 2005). In cultured neurons, expression of exogenous NLGN1 promotes excitatory synapse formation (Dean et al., 2003; Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). NLGN1 plays an important role in governing the synaptic distribution of N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDAR) (Barrow et al., 2009; Jung et al., 2010; Budreck et al., 2013). Deletion of NLGN1 decreases the currents mediated by NMDAR (Chubykin et al., 2007; Espinosa et al., 2015; Zhang and Sudhof, 2016), impairs NMDAR-dependent long-term potentiation (LTP) (Jung et al., 2010), and causes deficits in spatial learning and memory (Kim et al., 2008; Blundell et al., 2010).

In spinal cord dorsal horn, the excitatory glutamatergic synapses between primary afferent central terminals and superficial dorsal horn neurons are the primary sites for conveying sensory information from periphery to higher levels of central regions. These glutamatergic synapses have been shown to undergo a series of plastic changes after peripheral tissue or nerve injuries (Sandkuhler, 2009; Luo et al., 2014). One of the significant changes is the sustained increase in the synaptic concentration of NMDAR, especially those containing GluN2B subunit (Wu et al., 2005; Iwata et al., 2007; Yang et al., 2009). Such a dynamic reorganization of synaptic NMDAR results in long-lasting modification of NMDAR-mediated synaptic responses and signaling

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Abbreviations: ACSF, artificial cerebrospinal fluid; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CFA, complete Freund's adjuvant; D-APV, D(−)-2-Amino-5-phosphonopentanoic acid; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Fyn (K296M), lysine-to-methionine mutant of Fyn at residue 296; KN-93, 2-[N-(2-hydroxyethyl)]-N-(4methoxybenzenesulfonyl)-amino-N-(4-chloro cinnamyl)-N-methylbenzylamine; LTP, long-term potentiation; NLGN1, Neuroligin 1; NLGNs, Neuroligins; NMDAR, N-methyl-D-aspartate-subtype glutamate receptor; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-D]pyrimidine; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; SDS, sodium dodecyl sulfate; SFKs, Src-family protein tyrosine kinase; SNL, spinal nerve ligation.

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transductions, and is believed to be an important mechanism underlying chronic inflammatory and neuropathic pain (Gogas, 2006; Qu et al., 2009; Bourinet et al., 2014). However, it is still unclear whether the reorganization of NMDAR requires the involvement of NLGN1.

NLGN1 exhibits dynamic changes in cellular localization in response to ongoing neuronal activity. Increased synaptic activity leads to the accumulation of NLGN1 on postsynaptic surface membrane (Gutierrez et al., 2009; Schapitz et al., 2010). Several studies have investigated the structural determinants for NLGN1 trafficking and localization. The short intracellular domain of NLGN1 contains several serine and threonine residues that are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Bemben et al., 2014). The CaMKII-mediated phosphorylation of NLGN1 at T739 is governed by neuronal activity and promotes the forward trafficking of NLGN1 to the plasma membrane (Bemben et al., 2014). In addition to serine/threonine phosphorylation, tyrosine phosphorylation of NLGN1 blocks its interaction with gephyrin, but increases its binding to PSD-95 (Giannone et al., 2013). Therefore, the activity-driven phosphorylation is a key regulatory mechanism for synaptic tethering of NLGN1. In spinal dorsal horn, both CaMKII and Src-family protein tyrosine kinases (SFKs) member Fyn have been shown to modulate NMDAR function during inflammatory and neuropathic pain (Chen and Roche, 2007; Luo et al., 2008; Yang et al., 2011; Crown et al., 2012; Hildebrand et al., 2016). It is, however, poorly defined whether these kinases act through NLGN1.

NLGN1 are expressed throughout the gray matter of the spinal cord, especially in the pain-related superficial lumbar dorsal horn (Dolique et al., 2013). Spinal nerve ligation (SNL)-induced neuropathic pain has been shown to correlate with the increased NLGN1 interaction with PSD-95 and phosphorylated GluN2B without any effects on the protein expression of NLGN1 (Dolique et al., 2013; Lin et al., 2015), indicating the potential role of NLGN1 in NMDAR modification. Knockdown of NLGN1 prevents the SNL induced-mechanical allodynia (Lin et al., 2015). To characterize the possible contribution of NLGN1 to inflammatory pain, the present study analyzed the changes of NLGN1 expression after intraplantar injection of complete Freund's adjuvant (CFA). Our data revealed an activity-dependent recruitment of NLGN1 into synapses, which was essential for NMDAR hyperfunction and pain hypersensitivity.

EXPERIMENTAL PROCEDURES

Animals

Male adult C57BL/6J mice (22–25 g) and male Sprague–Dawley rats (80–100 g) were purchased from the Experimental Animal Center of Lanzhou University and housed in plastic cages with free access to food and water. The mice were habituated to the test environment for three days before any experiments were conducted. All experiments were approved by the Animal Care and Use Committee of Lanzhou University. Every effort was made to minimize the number of animals used. A total of 158 mice and 60 rats were used in this study.

Intrathecal injection and behavioral tests

The animals were held with a pelvic girdle. A 30-gauge needle was inserted into the L5–6 vertebrae (Hylden and Wilcox, 1980; Suo et al., 2013), and NMDARs' antagonist D(−)-2-Amino-5-phosphonopentanoic acid (D-APV; Sigma), CaMKII inhibitor 2-[N-(2-hydroxyethyl)]-N-(4methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93; Sigma), or SFKs inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2; Calbiochem, La Jolla, CA, USA) was injected slowly in a 5-μl volume. D-APV was dissolved in saline. KN-93 and PP2 were dissolved in dimethyl sulfoxide (DMSO), which were diluted to appropriate concentrations with saline before use. The final concentration of DMSO was less than 0.5%, which had no significant impact on the behavioral responses (Li et al., 2011). For Fyn inhibition, 2.5 μg of pCMV-SPORT6 vector encoding catalytically null Fyn(K296M) mutant was mixed with 5-μl lipofecter liposomal transfection reagent (Beyotime Institute of Biotechnology, Jiangsu, China), and was intrathecally injected (Yang et al., 2011). To establish the inflammatory pain model, complete Freund's adjuvant (CFA; 1 mg/ml heat-killed Mycobacterium tuberculosis; sigma) in 10-μl volumes was injected into the plantar surfaces of the hindpaws of mice. The thermal hyperalgesia was measured by exposing the plantar surfaces of hindpaws to a beam of radiant light, and the paw withdrawal latency (PWL) values were recorded (Wang et al., 2016; Suo et al., 2017). The mechanical allodynia was assessed by applying Von Frey's stimuli to the plantar surfaces of hindpaws. A series of eight Von Frey's filaments with approximately equal logarithmic incremental bending forces were chosen (log force: 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17; equivalent to 0.02, 0.03, 0.07, 0.17, 0.41, 0.69, 1.20 and 1.48 g, respectively). Beginning with filament 3.22, the filaments were applied perpendicularly to the plantar surface until bending. The rapid paw withdrawal indicated a positive response. The 50% paw withdrawal threshold (PWT) was calculated using the Up-Down method (Chaplan et al., 1994).

NLGN1 knockdown *in vivo*

Three small interfering RNA (siRNA) targeting mouse NLGN1 gene was designed and synthesized by Genepharma (Shanghai, China). The sequences were as follows: siRNA1 (5′-GGUUUCAUCAUACGUCCAATT-3′ and 5′-UUGGACGUAUGAUGAAACCTT-3′); siRNA2 (5′-CCAGCUGGGCUGUUAGUUUTT-3′ and 5′-AAACUAACAGCCCAGCUGGTT-3′); siRNA3 (5′-CCAGAUGCAUGUGGCCAA ATT-3′ and 5′-UUUGGCCACAUGCAUCUGGTT-3′). A scrambled sequence (scrambled siRNA; Genepharma) was also synthesized as negative control. For spinal delivery (Tan et al., 2005; Njoo et al., 2014), the branched polyethyleneimine (PEI; Sigma) was mixed with siRNA with the ratio of PEI nitrogen to siRNA phosphate being 8:1. The mixture was allowed to stand at room temperature for 30 min before intrathecal