Ubiquitination and functional modification of GluN2B subunit–containing NMDA receptors by Cbl-b in the spinal cord dorsal horn

Zi-Yang Zhang, Hu-Hu Bai, Zhen Guo, Hu-Ling Li, Xin-Tong Diao, Tian-Yu Zhang, Lin Yao, Juan-Juan Ma, Zheng Cao, Yin-Xia Li, Xue Bai, Hai-Kun Chen, Zhan-Wei Suo, Xian Yang, Xiao-Dong Hu*

N-methyl-D-aspartate (NMDA) glutamate receptors (NMDARs) containing GluN2B subunits are prevalent early after birth in most brain regions in rodents. Upon synapse maturation, GluN2B is progressively removed from synapses, which affects NMDAR function and synaptic plasticity. Aberrant recruitment of GluN2B into mature synapses has been implicated in several neuropathologies that afflict adults. We found that the E3 ubiquitin ligase Cbl-b was enriched in the spinal cord dorsal horn neurons of mice and rats and suppressed GluN2B abundance during development and inflammatory pain. Cbl-b abundance increased from postnatal day 1 (P1) to P14, a critical time period for synapse maturation. Through its N-terminal tyrosine kinase binding domain, Cbl-b interacted with GluN2B. Ubiquitination of GluN2B by Cbl-b decreased the synaptic transmission mediated by GluN2B-containing NMDARs. Knocking down Cbl-b in vivo during P1 to P14 led to sustained retention of GluN2B at dorsal horn synapses, suggesting that Cbl-b limits the synaptic abundance of GluN2B in adult mice. However, peripheral inflammation induced by intraplantar injection of complete Freund’s adjuvant resulted in the dephosphorylation of Cbl-b at Tyr363, which impaired its binding to and ubiquitylation of GluN2B, enabling the reappearance of GluN2B-containing NMDARs at synapses. Expression of a phosphomimic Cbl-b mutant in the dorsal horn suppressed both GluN2B-mediated synaptic currents and manifestations of pain induced by inflammation. The findings indicate a ubiquitin-mediated developmental switch in NMDAR subunit composition that is dysregulated by inflammation, which can enhance nociception.

INTRODUCTION

Casitas B-lineage lymphoma b (Cbl-b) is an E3 ubiquitin ligase that contains an N-terminal tyrosine kinase binding (TKB) domain, a helical linker, a RING finger, several proline-rich domains (PRDs), and a C-terminal ubiquitin-associated (UBA) domain (1–4). The E3 ubiquitin ligase activity of Cbl-b relies on the RING finger domain, which recruits E2 ubiquitin-conjugating enzymes and is responsible for the transfer of ubiquitin molecules from E2 enzymes to the lysine residues on Cbl-b–associated substrates (4). The RING finger is usually concealed by an intramolecular interaction (5). The activity-dependent phosphorylation of Tyr363 within the helical linker can disrupt the intramolecular interaction and lead to Cbl-b activation (5). The TKB domain is proposed to determine the substrate specificity of Cbl-b (3, 6). Through interacting with a line of transmembrane proteins, the TKB domain directs the cytoplasmic Cbl-b to plasma membrane, where the protein substrates are ubiquitinated. The C-terminal PRD and UBA domains have been implicated in the scaffolding of several signaling and adaptor proteins as well as endocytic machinery (6, 7), conferring to Cbl-b an important role in the signaling transduction and diverse pathophysiological processes such as tumorigenesis and diabetes (3, 8, 9).

To date, Cbl-b has been well characterized in the inhibition of receptor tyrosine kinase (RTK) signaling (3). Once ubiquitinated by Cbl-b, activated RTKs are sorted into endocytic pathways and subjected to lysosomal or proteasomal degradation (4). In the central nervous system, ubiquitination represents an important way to alter the synaptic transmission and plasticity (2, 10). Previous studies have indicated that Cbl-b is concentrated in several hippocampal regions (2). Cbl-b deletion enhances the long-term memory and short-term plasticity in mice (2). Nevertheless, the means by which Cbl-b achieves the synaptic modification are not yet well defined.

N-methyl-D-aspartate (NMDA)–subtype glutamate receptor (NMDAR) is composed of obligatory GluN1 subunit and regulatory GluN2 (GluN2A to GluN2D) or GluN3 subunits. GluN2B-containing NMDARs are prevalent during synaptogenesis but are gradually replaced by GluN2A-containing NMDARs with synapse maturation (11–13). This subunit switch alters the biological and pharmacological properties of NMDAR and regulates NMDAR-dependent synaptic plasticity. In pain-related spinal cord dorsal horn of adult animals, the redistribution of GluN2B into mature synapses after peripheral tissue or nerve injuries enhances synaptic GluN2B/GluN2A ratio (14), resulting in the amplified nociceptive synaptic transmission. In this study, we found a specific interaction of Cbl-b with GluN2B subunit and demonstrated that Cbl-b binding to GluN2B was involved in the modification of NMDAR subunit composition during development and inflammatory pain.

RESULTS

Cbl-b is distributed at spinal cord synapses

The Cbl-b protein has been shown to locate at hippocampal regions (2). Whether this E3 ubiquitin ligase was expressed in pain-related spinal cord remained uncharacterized. By performing immunohistochemistry in spinal cord slices, we found that Cbl-b was enriched in the dorsal horn and coincident with neuronal marker NeuN (Fig. 1A). Cbl-b did not colocalize with microglia marker OX-42 (Fig. 1A) or
Cbl-b specifically interacts with NMDAR subunit GluN2B

NMDARs and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid–subtype glutamate receptors (AMPARs) are two major mediators of nociceptive synaptic transmission in the spinal dorsal horn. To identify the possible substrates of Cbl-b, we conducted coinmunoprecipitation from lysates of spinal dorsal horns from adult mice. Our data showed that an antibody to Cbl-b precipitated NMDAR subunits GluN2B and GluN1 (Fig. 2A), whereas NMDAR subunit GluN2A and AMPAR subunit GluA2 were undetectable in Cbl-b precipitates (Fig. 2A). Reciprocally, the antibody against GluN2B stably pulled down Cbl-b (Fig. 2B). As well, C-Cbl, the Cbl-b homology protein that is also present in the spinal cord and is involved in pain processing (15), did not interact with GluN2B (Fig. 2B). To examine whether Cbl-b directly bound to GluN2B, we cotransfected human embryonic kidney (HEK) 293T cells with GluN2B, GluN1, and Myc-tagged Cbl-b (Myc-Cbl-b), finding that anti-GluN2B antibody faithfully precipitated Myc-Cbl-b (Fig. 2C). Such an interaction was not observed between GluN2A and Myc-Cbl-b (Fig. 2D).

Cbl-b contains several protein–protein interaction domains (Fig. 2E). To identify which region of Cbl-b was responsible for GluN2B binding, we cotransfected GluN2B and GluN1 in HEK293T cells along with several Myc-tagged Cbl-b constructs (Fig. 2E). The results showed that Myc-Cbl-b(1–344), which encompassed only the TKB domain (Fig. 2E), pulled down GluN2B as with Myc-tagged full-length Cbl-b (Fig. 2F). Myc-Cbl-b(345–426), which harbored the helical linker and RING finger (Fig. 2E), did not interact with GluN2B (Fig. 2F). Myc-Cbl-b(427–938), which contained the C-terminal region of Cbl-b (Fig. 2E), also failed to precipitate GluN2B (Fig. 2F). Therefore, the TKB domain was the key determinant for GluN2B binding.

Cbl-b increases the ubiquitination of GluN2B and decreases its surface expression

We next examined whether the E3 ligase activity of Cbl-b ubiquitinated GluN2B. In HEK293T cells, the ectopically expressed Cbl-b remarkably enhanced the ubiquitination level of GluN2B when compared to empty vector (Fig. 3A), whereas the Cbl-b homology C-Cbl had no significant effect (Fig. 3B). The expression of Cbl-b did not influence GluN2A ubiquitination (Fig. 3C), confirming the specific targeting of Cbl-b to GluN2B. Our data showed that GluN2B was conjugated by Cbl-b with Lys63-linked ubiquitin (Fig. 3D) and not Lys48-linked ubiquitin chains (Fig. 3E). Neuronal precursor cell–expressed developmentally down-regulated–4 type 1 (NEDD4-1) and Mind bomb-2, another two E3 ligases that have been shown to ubiquitinate NMDAR (16, 17), generated no detectable effects on GluN2B under our conditions (Fig. 3, D and E), providing additional evidence for the specificity of Cbl-b in the modification of GluN2B.

To test whether Cbl-b modified GluN2B in cultured neurons, we used short hairpin RNAs (shRNAs) to knock down endogenous Cbl-b. Of four designed shRNAs, shRNA-2046 potently reduced the protein level of Cbl-b (Fig. 3F). The Cbl-b knockdown did not inhibit the expression of GluN2B (Fig. 3F). We therefore infected neurons at DIV12-14 with shRNA-2046 (denoted sh-Cbl-b) and examined GluN2B ubiquitination after 3 days. Western blot of GluN2B immunoprecipitates showed that sh-Cbl-b noticeably reduced the attachment of Lys63-linked ubiquitin to GluN2B (Fig. 3G), suggesting that endogenous Cbl-b was tonically involved in the ubiquitination of GluN2B. Simultaneous expression of the shRNA-resistant Cbl-b construct rescued the GluN2B ubiquitination (Fig. 3G), excluding the off-target effects of sh-Cbl-b.

Lys63-linked ubiquitin represents an important signal for the endocytosis of transmembrane proteins (18). To examine the effect of Cbl-b on surface GluN2B, we treated neurons at DIV12-14 with sh-Cbl-b and conducted immunostaining with anti-GluN2B N-terminal antibody under nonpermeable conditions. Compared with negative control shRNA, sh-Cbl-b increased the immunofluorescence intensities of GluN2B on plasma membrane (Fig. 3H). The coexpression of Cbl-b with sh-Cbl-b repressed surface GluN2B content to control level (Fig. 3H), suggesting that ubiquitin modification promoted by Cbl-b—directly or indirectly—decreased GluN2B distribution on plasma membrane.

Cbl-b limits GluN2B synaptic transmission during development

GluN2B is prevalent at glutamatergic synapses by the first postnatal week. With synaptic development, GluN2B receptors are gradually
dispersed out of synapses and replaced by GluN2A subunit-containing NMDAR (J1). We noted that the protein level of Cbl-b progressively increased from postnatal day 1 (P1) on (Fig. 4A), which reached peak at about P7 to P14 (Fig. 4A). Coincident with the increased Cbl-b expression was the enhanced conjugation of Lys<sup>63</sup>-linked ubiquitin to GluN2B at P14 (Fig. 4B). At P21, GluN2B was still attached to Lys<sup>63</sup>-linked ubiquitin in spinal dorsal horn, albeit to a less degree when compared to immature tissues at P14 (Fig. 4B).

To test whether the enhanced Cbl-b expression contributed to the developmental decline of synaptic GluN2B receptors, we intrathecally injected lentivirus encoding sh-Cbl-b in mice at P5 and P6 and recorded NMDAR-mediated excitatory postsynaptic currents (EPSCs) after 10 to 12 days. Compared to negative control shRNA, sh-Cbl-b decreased spinal Cbl-b expression (Fig. 4C) and prolonged the decay time of NMDAR-EPSCs (Fig. 4D), suggesting that Cbl-b knockdown allowed the retention of more GluN2B at synapses. The rescue of Cbl-b expression (Fig. 4C) shortened the decay time of NMDAR-EPSCs to control level (Fig. 4D). We next evaluated the contribution of GluN2B to NMDAR synaptic currents by extracellular perfusion of ifenprodil (3 μM), a selective GluN2B receptor antagonist. In neurons expressing sh-Cbl-b, bath application of ifenprodil decreased the amplitudes of NMDAR-EPSCs to a greater degree than in neurons expressing control shRNA (Fig. 4E), confirming that Cbl-b knockdown enhanced the GluN2B components of synaptic responses. The input-output curves illustrated that synaptic GluN2B accumulation increased the amplitudes of NMDAR-EPSCs in neurons expressing sh-Cbl-b relative to control shRNA (Fig. 4F). The enhanced ifenprodil sensitivity (Fig. 4E) and NMDAR-EPSC amplitudes by sh-Cbl-b (Fig. 4F) were repressed by the coexpressed Cbl-b construct. Western blot showed that the developmental decrease of synaptic GluN2B from P7 to P21 was blocked by sh-Cbl-b and rescued by simultaneous expression of Cbl-b (Fig. 4G). The synaptic accumulation of GluN2A with development was unaltered by sh-Cbl-b or sh-Cbl-b and Cbl-b (Fig. 4G). These data suggested that Cbl-b was involved in the removal of GluN2B from synapses during development.

**Peripheral inflammation reduces the binding affinity of Cbl-b for GluN2B**

As presented above, Cbl-b underwent an age-dependent alteration in its protein level after birth (Fig. 4A). However, Cbl-b was still detectable in adulthood and stably interacted with GluN2B (Fig. 2, A and B). To reveal the function of Cbl-b in adult mice, we knocked down Cbl-b by intrathecal injection of lentivirus encoding sh-Cbl-b. Our data showed that the GluN2B amount on plasma membrane was significantly enhanced by sh-Cbl-b, which could be repressed to control level by simultaneously expressed Cbl-b (Fig. 5, A and B). The total GluN2B content was unaltered by sh-Cbl-b or sh-Cbl-b/Cbl-b (Fig. 5, A and C). We did not detect any changes in the surface and total protein levels of GluN2A and GluA2 subunit after Cbl-b knockdown (Fig. 5, A to C). These data suggested that Cbl-b was constitutively active in the inhibition of GluN2B surface expression in mature spinal cord.

Convincing evidence has indicated the importance of synaptic GluN2B hyperfunction in inflammatory pain. To test the possible role of Cbl-b in nociceptive signal processing, we induced inflammatory pain by intraplantar injection of complete Freund's adjuvant (CFA) in adult mice. The paw withdrawal thresholds (PWTs) to innocuous mechanical stimulation and paw withdrawal latencies (PWLS) to thermal stimulation were markedly decreased 1 day after CFA injection (Fig. 5D). At this time, we examined the protein level of Cbl-b in lysates of spinal dorsal horn, finding that peripheral inflammation did not change the total expression of Cbl-b (Fig. 5E). However, the tyrosine phosphorylation of Cbl-b, a biological correlate of the E3 ligase activity of Cbl-b, was significantly decreased in the inflamed mice relative to saline-injected control ones (Fig. 5, F and G), suggesting that inflammatory lesion led to the inhibition of spinal Cbl-b activity. Intrathecal application of NMDAR antagonist d(-)-2-amino-5-phosphonopentanoic acid (D-APV; 0.5 μg) or protein tyrosine phosphatase (PTP) inhibitor bpV(phen) [potassium bisperoxo(1,10-phenanthroline)oxovanadate (V); 0.5 μg] reversed the reduction of Cbl-b tyrosine phosphorylation in inflamed mice (Fig. 5, F and G), indicating that NMDAR-stimulated certain PTPs might dephosphorylate Cbl-b during inflammatory pain. Cbl-b dephosphorylation was associated with the reduced binding to GluN2B (Fig. 5, H and I). Inhibition of NMDAR or PTP activities totally resumed GluN2B/Cbl-b interaction in CFA mice (Fig. 5, H and I).

To further characterize the activity-dependent Cbl-b dephosphorylation, we treated the cultured neurons expressing Myc-Cbl-b with NMDA (50 μM) for 10 min. Consistent with the observations in vivo (Fig. 5F), NMDAR activation reduced the phosphorylation level of Myc-Cbl-b (Fig. 5, J and K). There is a key regulatory tyrosine residue

**Fig. 2. Specific interaction of Cbl-b with the GluN2B subunit.** (A and B) Cbl-b (A) or GluN2B immunoprecipitates (B) from spinal cord dorsal horn of adult mice were probed with the antibodies indicated on the left of panels. n = 6 experiments per group. IgG, immunoglobulin G; IP, immunoprecipitation. (C and D) Myc-Cbl-b was cotransfected in HEK293T cells with GluN1 and either GluN2B (C) or GluN2A (D) and immunoprecipitated for the latter, respectively. n = 6 experiments per group. (E and F) Four Myc-tagged Cbl-b constructs were generated (E) and cotransfected with GluN2B and GluN1 in HEK293T cells. Myc immunoprecipitates were immunoblotted (IB) for GluN2B (F, top). Total cell lysates (TCL) were immunoblotted for GluN2B (F, middle) and Myc (F, bottom). n = 6 experiments.
Fig. 3. Cbl-b enhanced the ubiquitination and reduced the surface expression of GluN2B. (A and B) GluN2B immunoprecipitates from HEK293T cells coexpressing GluN1, ubiquitin (Ubi), and Myc-Cbl-b (A), or Myc-C-Cbl (B) were immunoblotted for ubiquitin. The TCLs were immunoblotted for Myc and GluN2B. ***P < 0.005 relative to control cells (Mann-Whitney U test). n = 6 experiments per group. (C) Effect of Myc-Cbl-b on GluN2A ubiquitination in HEK293T cells cotransfected with GluN1 and ubiquitin. n = 6 experiments. (D and E) Myc-Cbl-b or hemagglutinin (HA)-tagged NEDD4-1 or Mind bomb-2 (Mib2) was transfected into HEK293T cells along with GluN2B, GluN1, and ubiquitin. GluN2B immunoprecipitates were immunoblotted for Lys 63-linked ubiquitin [Lys 63-Ubi (D)] or Lys 48-linked ubiquitin [Lys 48-Ubi (E)]. ***P < 0.005, one-way ANOVA with post hoc Tukey’s test. n = 6 experiments per group. (F) Four shRNAs against Cbl-b and negative control shRNA (NC) were transfected in cultured spinal neurons. Cell lysates were immunoblotted for Cbl-b, GluN2B, and β-actin after 3 days. *P < 0.05 and **P < 0.01, one-way ANOVA with post hoc Tukey’s test. n = 6 experiments. (G) Effects of Cbl-b knockdown by shRNA-2046 (sh-Cbl-b), and rescue expression with shRNA-resistant Cbl-b, on the conjugation of Lys 63-linked ubiquitin to GluN2B in cultured spinal neurons. ***P < 0.005, one-way ANOVA with post hoc Tukey’s test. n = 6 experiments. (H) Effects of sh-Cbl-b and sh-Cbl-b/Cbl-b on the surface expression of GluN2B in spinal neuron cultures. The neurons were infected at DIV12-14, and the surface GluN2B (red) and intracellular GFP (green) were immunostained after 3 days. ***P < 0.005, one-way ANOVA with post hoc Tukey’s test. n = 30 neurons per group. Scale bar, 10 µm. All data are means ± SEM.
Fig. 4. Cbl-b promotes the developmental decline of synaptic GluN2B receptors. (A) Cbl-b contents in the whole-cell lysates of spinal cord dorsal horn of mice at postnatal days 1 to 21 (P1 to P21). ***P < 0.005, one-way ANOVA with post hoc Tukey’s test. n = 8 experiments. (B) GluN2B immunoprecipitates from spinal cord dorsal horn were immunoblotted for Lys63-linked ubiquitin at P1, P14, and P21. ***P < 0.005 relative to P1; one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. (C) Mice at P5 and P6 were intrathecally injected with lentivirus encoding negative control shRNA, sh-Cbl-b, or sh-Cbl-b- and shRNA-resistant Cbl-b. Cbl-b protein levels in spinal cord dorsal horn were examined after 10 to 12 days. Control mice received saline injection. *P < 0.05 and ***P < 0.005, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. (D) Effect of Cbl-b knockdown and of rescue expression with shRNA-resistant Cbl-b, on the decay time constant of NMDAR-mediated excitatory postsynaptic currents (EPSCs) recorded on spinal dorsal horn neurons. The peak-scaled EPSCs were also shown. *P < 0.05, one-way ANOVA and post hoc Tukey’s test. n = 6 neurons per group. (E) The sensitivity of NMDAR-EPSCs to ifenprodil in spinal cord dorsal horn neurons expressing negative control shRNA, sh-Cbl-b, or sh-Cbl-b/Cbl-b. The horizontal bar indicated the period of extracellular ifenprodil perfusion. The original top and bottom traces (inset), respectively, were taken at the time points indicated by numbers 1 and 2 on the top plot. F(68, 510) = 1.594, P < 0.005 (repeated measurement). n = 6 neurons per group. (F) Input-output curves of NMDAR-EPSCs in spinal cord dorsal horn neurons expressing negative control shRNA, sh-Cbl-b, or sh-Cbl-b/Cbl-b. The horizontal bar indicated the period of extracellular ifenprodil perfusion. The original top and bottom traces (inset), respectively, were taken at the time points indicated by numbers 1 and 2 on the top plot. F(8, 60) = 2.862, P < 0.01 (repeated measurement). Post hoc Bonferroni tests showed significant differences at 2 mV (*P < 0.05), n = 6 neurons per group. (G) Effect of Cbl-b knockdown and rescue on the developmental decrease of GluN2B abundance (blots, left) in the PSD fraction of spinal cord dorsal horn of mice from P7 to P21. Graph showed the percentage changes of synaptic GluN2B (middle) or GluN2A (right). *P < 0.05, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. All data are means ± SEM.
Fig. 5. Cbl-b dephosphorylation by peripheral inflammation disturbs the GluN2B/Cbl-b interaction in the spinal dorsal horn of adult mice. (A to C) Effect of Cbl-b knockdown and rescue on GluN2B (and GluN2A and GluA2) surface expression in the spinal cord dorsal horn of adult mice. Surface biotinylation assays were conducted at days 10 to 14 after intrathecal injection of lentivirus encoding negative control shRNA, sh-Cbl-b or sh-Cbl-b and Cbl-b (A). Graph illustrated the percentage changes of (B) surface and (C) total protein levels in the TCLs. *P < 0.05 and **P < 0.01, one-way ANOVA and post hoc Tukey’s test. (D) Paw withdrawal thresholds (PWTs) and paw withdrawal latencies (PWLs) at day 1 after saline or complete Freund’s adjuvant (CFA) injection into the plantar surfaces of hindpaws of mice. ***P < 0.005, Mann-Whitney U test. n = 6 mice per group. (E) Total Cbl-b protein levels in spinal cord dorsal horn of mice at day 1 after intraplantar injection of CFA or saline. n = 6 experiments. (F and G) CFA caused Cbl-b dephosphorylation through NMDAR/protein tyrosine phosphatase (PTP) signaling in spinal cord dorsal horn of adult mice. One day after intraplantar CFA injection, the NMDAR antagonist D-APV or the PTP inhibitor bpV(phen) (each 0.5 μg) was spinally applied for 30 min before Cbl-b immunoprecipitates were immunoblotted for PY20. *P < 0.05 and ***P < 0.005, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. (H and I) Coimmunoprecipitation (co-IP) for the GluN2B/Cbl-b interaction in the spinal cord dorsal horn of mice 1 day after CFA injection into the plantar surface. D-APV or bpV(phen) was spinally applied for 30 min before co-IP. **P < 0.01 and ***P < 0.005, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. (J and K) Pulldown and blotting for phosphorylated Cbl-b (at Tyr363) using PY20 in cultured spinal neurons expressing Myc-Cbl-b or its mutant Cbl-b(Y363F) and treated with NMDA (50 μM) for 10 min. *P < 0.05, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. (L and M) Effect of Tyr363 (de)phosphorylation on the binding affinity of Cbl-b for GluN2B in cultured spinal neurons transfected with Myc-tagged Cbl-b, Cbl-b(Y363E), or Cbl-b(Y363F) for 3 days. *P < 0.05 and ***P < 0.005, one-way ANOVA and post hoc Tukey’s test. n = 6 experiments. All data are means ± SEM.
(Tyr\(^{363}\)) within the helical linker of Cbl-b, whose dephosphorylation has been shown to impede the access of E2 ubiquitin-conjugating enzymes to the RING domain of Cbl-b\(^{5}\). We found that mutation of Tyr\(^{363}\) to phenylalanine (Y363F) abolished the regulatory effect of NMDAR on Cbl-b (Fig. 5, J and K), indicating that Tyr 363 was the major dephosphorylation site by NMDAR signaling.

To test whether Tyr\(^{363}\) phosphorylation influenced Cbl-b interaction with GluN2B, we substituted Tyr\(^{363}\) with glutamic acid (Y363E) to generate a Myc-tagged, phosphomimicking Cbl-b mutant. In the transfected neurons, antibody to GluN2B immunoprecipitated more Myc-Cbl-b(Y363E) than Myc-Cbl-b(wild-type) (Fig. 5, L and M). By contrast, the phosphorylation-deficient Myc-Cbl-b(Y363F) mutant, in which Tyr\(^{363}\) was substituted with phenylalanine, interacted with GluN2B to a less extent than Myc-Cbl-b or Myc-Cbl-b(Y363E) (Fig. 5, L and M). These data suggested that Tyr\(^{363}\) dephosphorylation disrupted Cbl-b interaction with GluN2B.

Peripheral inflammation reduces Cbl-b–mediated GluN2B ubiquitination

Because Cbl-b remained active in the inhibition of GluN2B surface expression in adult spinal cord (Fig. 5A), we wanted to know whether the dissociation of Cbl-b/GluN2B complex allowed for GluN2B hyperfunction during the sensitized pain states. In adult mice, GluN2B exhibited a basal ubiquitination level (Fig. 6A). CFA injection, however, significantly decreased GluN2B ubiquitination when compared to saline control (Fig. 6, A and B). Spinal treatment with either D-APV or bpV(phen) increased the ubiquitinated GluN2B levels (Fig. 6, A and B), indicating a close relationship between the reduced GluN2B ubiquitination and pain hypersensitivity.

To determine whether the reduced GluN2B ubiquitination resulted from Cbl-b inactivation, we intrathecally injected adenovirus encoding green fluorescent protein (GFP) and the active Cbl-b(Y363E) mutant at 24 hours after CFA. Double immunostaining illustrated
Overexpression of Cbl-b(Y363E) attenuates inflammatory pain

Because Cbl-b(Y363E) repressed GluN2B-mediated nociceptive transmission, we investigated whether this mutant alleviated the painful behaviors. For this purpose, we intrathecally administered adenovirus encoding GFP or Cbl-b(Y363E) at 24 hours after intradermal CFA injection in rats. Compared to those in GFP rats, the PWT (Fig. 7A) and PWL values (Fig. 7B) were significantly enhanced at day 2 after Cbl-b(Y363E) expression. The analgesic action reached maximum at day 4 after viral injection and lasted for at least 5 days (Fig. 7, A and B).

Intradermal formalin injection can evoke biphasic spontaneous inflammatory pain. We virally expressed GFP or Cbl-b(Y363E) for 3 days before formalin injection into the left hindpaws. The results showed that Cbl-b(Y363E) attenuated both the first-phase (Fig. 7, C and D) and second-phase responses (Fig. 7, C and E) when compared to GFP control. These data suggested that the enhancement of Cbl-b activity generated an effective analgesic action against inflammatory pain.

**DISCUSSION**

Cumulative evidence has mounted that the synaptic efficacy is reduced by the ubiquitination of glutamate receptors. Several E3 ubiquitin ligases, including RING finger protein 167 and NEDD4-1, have been shown to ubiquitate AMPAR, leading to the reduced glutamatergic neurotransmission (19–21). NEDD4-1 and Mind bomb-2 are two E3 ligases that ubiquitinate NMDAR GluN2D subunit and tyrosine-phosphorylated GluN2B subunit, respectively (16, 17). Our data showed that NEDD4-1 did not increase GluN2B ubiquitination, consistent with previous report that NEDD4-1 did not interact with GluN2B (17). Mind bomb-2 also failed to enhance GluN2B ubiquitination, possibly because of the low tyrosine phosphorylation of GluN2B in the absence of active Fyn kinase (16). The RING domain–containing E3 ligase Cbl-b has been implicated in the regulation of memory retention and synaptic plasticity in hippocampus (2). Here, we found that Cbl-b was located at spinal cord synapses and specifically interacted with GluN2B subunit. We provided evidence that Cbl-b–mediated GluN2B ubiquitination played an important role in the dynamic modification of GluN2B synaptic transmission during development and inflammatory pain.

The subunit switch from GluN2B- to GluN2A-containing NMDAR is widely regarded as an integral feature of synapse maturation (11–13). This process might involve the integral removal of GluN2B from synapses and subsequent accumulation of GluN2A on postsynaptic membrane. The activity-dependent GluN2B phosphorylation has been proposed to drive GluN2B endocytosis and accelerate the subunit switch (12). The current study illustrated an age-dependent expression of Cbl-b and a selective conjugation of Lys63-linked ubiquitin by Cbl-b to GluN2B. The Lys63-linked ubiquitin chains have been implicated in endocytotic processes, whereas Lys48-linked ubiquitin chains contribute to protein degradation (18). The shRNA-mediated Cbl-b knockdown inhibited GluN2B ubiquitination and enhanced GluN2B component of synaptic transmission, suggesting that ubiquitin modification of GluN2B by Cbl-b was involved in synaptic NMDAR subunit switch. Whether there is a relationship between GluN2B phosphorylation and ubiquitination during development requires further investigation.

In adulthood, the excitatory glutamatergic synapses predominately express GluN2A receptors (5). Our data showed that, at P21, the protein level of Cbl-b had declined from the peak values of P7 to P14. However, the remaining Cbl-b still interacted with GluN2B and the basal ubiquitination of GluN2B was stably detected. The shRNA knockdown of Cbl-b enhanced the surface expression of GluN2A. Our data illustrated an age-dependent increase in Cbl-b expression in the adult brain. This might occur in response to conditions that reduce GluN2B phosphorylation, such as chronic pain.

**Figure 7.** Spinal expression of Cbl-b(Y363E) inhibited inflammatory pain in rats. (A) Time-dependent changes of PWTs of rats after intrathecal injection of adeno-virus (AV) encoding GFP or Cbl-b(Y363E). The arrowhead and arrow indicated the time points when intradermal CFA and intrathecal virus were injected, respectively. Repeated-measurement ANOVA showed a significant time × group interaction [F(7, 70) = 13.697, P < 0.001]. Post hoc Bonferroni tests showed significant differences between groups at days 2 to 6; *P < 0.05, n = 6 rats per group. BL, baseline. (B) Time-dependent changes of PWLs of rats. F(7, 70) = 5.202, P < 0.001, repeated-measurement ANOVA. Post hoc Bonferroni tests showed significant differences between groups at days 1 to 6; *P < 0.05, n = 6 rats per group. (C to E) Effects of Cbl-b(Y363E) on formalin-induced spontaneous pain (C) in rats. Repeated-measures ANOVA showed a significant time × group interaction [F(11, 110) = 1.965, P = 0.039]. The adeno-virus encoding GFP or Cbl-b(Y363E) was intrathecally administered for 3 days before intradermal formalin injection. The total time spent by rats on licking/lifting the injured paws during the first phase (D) and second phase (E) were summarized. ***P < 0.005 and **P < 0.01 relative to GFP; Mann-Whitney U test. n = 6 rats per group. All data are means ± SEM.
GluN2B, suggesting that Cbl-b activity persisted in adulthood and worked to maintain synaptic NMDAR subunit composition by preventing GluN2B synaptic accumulation.

After peripheral tissue injury in adult animals, GluN2B receptors can be motivated to reenter into spinal cord synapses, leading to GluN2B-dependent pain sensitization (22). Our data demonstrated that peripheral inflammation disrupted Cbl-b interaction with GluN2B in spinal dorsal horn. The impaired ability of Cbl-b to ubiquitinate GluN2B resulted in the potentiated synaptic nociceptive transmission mediated by GluN2B.

The N-terminal region of Cbl-b is thought to inhibit the E3 ligase activity of Cbl-b by forming an intramolecular interaction with RING domain (5). Tyrosine phosphorylation of Tyr363 within the helical linker can disrupt the intramolecular interaction so that the N-terminal region and RING domain are exposed simultaneously (5). The free N-terminal region and RING domain then bind to the substrates and E2 ubiquitin-conjugating enzymes, respectively. Our data showed that amino acid sequence of Cbl-b to the substrates and E2 ubiquitin-conjugating enzymes, respectively. Our data showed that the amino acid sequence of Cbl-b is involved in the inhibition of E3 ligase activity of Cbl-b.

The N-terminal region of Cbl-b is a potential substrate for tyrosine kinase and tyrosine phosphatase. The tyrosine kinase activity of Cbl-b is inhibited by phosphorylation at Tyr363, which is catalyzed by PTPases. The tyrosine phosphatase activity of Cbl-b is inhibited by dephosphorylation at Tyr363, which is catalyzed by Cbl-b.

The N-terminal region of Cbl-b is a potential substrate for tyrosine kinase and tyrosine phosphatase. The tyrosine kinase activity of Cbl-b is inhibited by phosphorylation at Tyr363, which is catalyzed by PTPases. The tyrosine phosphatase activity of Cbl-b is inhibited by dephosphorylation at Tyr363, which is catalyzed by Cbl-b.

The N-terminal region of Cbl-b is a potential substrate for tyrosine kinase and tyrosine phosphatase. The tyrosine kinase activity of Cbl-b is inhibited by phosphorylation at Tyr363, which is catalyzed by PTPases. The tyrosine phosphatase activity of Cbl-b is inhibited by dephosphorylation at Tyr363, which is catalyzed by Cbl-b.

The N-terminal region of Cbl-b is a potential substrate for tyrosine kinase and tyrosine phosphatase. The tyrosine kinase activity of Cbl-b is inhibited by phosphorylation at Tyr363, which is catalyzed by PTPases. The tyrosine phosphatase activity of Cbl-b is inhibited by dephosphorylation at Tyr363, which is catalyzed by Cbl-b.

The N-terminal region of Cbl-b is a potential substrate for tyrosine kinase and tyrosine phosphatase. The tyrosine kinase activity of Cbl-b is inhibited by phosphorylation at Tyr363, which is catalyzed by PTPases. The tyrosine phosphatase activity of Cbl-b is inhibited by dephosphorylation at Tyr363, which is catalyzed by Cbl-b.
standard calcium phosphate precipitation method. Thirty-six to 48 hours after transfection, the cells were harvested for analysis.

The primary neurons were cultured as described previously (26). Briefly, the mouse pups (P1) were decapitated, and the dorsal quadrants of spinal cords were dissected into ice-cold Hanks’ balanced salt solution (HBSS) supplemented with 10 mM Hepes. The spinal tissues were cut into small strips and digested with papain (2 mg/ml) in HBSS/Hepes solution for 20 to 30 min at 37°C. The digestion action was terminated by mixture with DMEM containing 10% heat-inactivated FBS. After trituration, the neurons were harvested by centrifugation at 1000 g for 5 min and resuspended in neurobasal medium supplemented with 2% FBS, 2% B27, 2% heat-inactivated horse serum, 1% penicillin/streptomycin, and 2 mM l-glutamine. The neurons were plated onto poly-d-lysine (0.1 mg/ml)–coated coverslips. Lipo6000 Transfection Reagent (Beyotime Institute of Biotechnology, Jiangsu, China) was used to transfect the cultured neurons according to the manufacturer’s instructions.

**Immunohistochemistry**

The rats were anesthetized deeply by intraperitoneal injection of sodium pentobarbital (90 to 120 mg/kg) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde. The spinal cords were postfixed for 4 hours in 4% paraformaldehyde and cryoprotected overnight at 4°C in PBS containing 30% sucrose (27). The frozen tissues were sectioned (14-μm thickness) with a cryostat at −20°C.

**Subcellular fractionation**

The mice were deeply anesthetized with sodium pentobarbital. After a laminectomy, the L4-L5 spinal cord was isolated into ice-cold artificial cerebrospinal fluid (ACSF; 119.0 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl$_2$, 1.3 mM MgCl$_2$, 1.0 mM Na$_2$PO$_4$, 26.0 mM NaHCO$_3$, and 11.0 mM d-glucose, bubbled with 95% O$_2$ + 5% CO$_2$, pH 7.4). The transverse slices (300-μm thickness) were cut with a vibratome. The dorsal quadrants were microdissected out and incubated for 45 min in ice-cold ACSF containing 100 μM Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific, Rockford, IL, USA), followed by brief washes with NH$_4$Cl (50 mM)–containing ACSF. After three washes with normal ACSF, the minislices were lysed in 20 mM tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 5 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 14,000g for 10 min, the supernatant was collected and incubated with Streptavidin UltraLink Resin (Thermo Fisher Scientific) overnight at 4°C under gentle rotation (25). The resin was washed three times with the lysis buffer and boiled in SDS sample buffer.

**Immunocytochemistry**

To assay the synaptic localization of Cbl-b (25), the neurons at DIV12-14 were fixed for 10 min in PBS containing 4% paraformaldehyde and 4% sucrose at room temperature. The neurons were then permeabilized and blocked for 30 min with 0.25% Triton X-100 and 10% NGS in PBS, followed by incubation with rabbit anti-Cbl-b antibody and mouse anti-PSD-95 antibody (Affinity BioReagents, Golden, CO, USA) for 2 hours at room temperature. After five washes with PBS, the labeled proteins were visualized by incubation with Alexa Fluor 488–conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit secondary antibodies at room temperature for 2 hours before image capture. The experiments were conducted and analyzed blindly.

**Immunoprecipitation and Western blot**

The animals were deeply anesthetized with sodium pentobarbital, and the spinal cords were removed into ice-cold ACSF. The dorsal quadrants of L4-L5 spinal segments were homogenized in the lysis buffer containing 10.0 mM tris-HCl (pH 7.6), 320.0 mM sucrose, 5.0 mM EDTA, and protease/phosphatase inhibitors (10.0 mM NaF, 1.0 mM Na$_3$VO$_4$, 1.0 mM PMSF, and 1.0 mg/ml each of aprotinin, chymostatin, leupeptin, antipain, and pepstatin). The homogenates were centrifuged at 1000g for 10 min at 4°C to remove the nuclei and large debris (P1). The supernatant (S1) was further centrifuged at 10,000g for 15 min to yield the crude synaptosomal fraction (P2). To isolate PSD, the P2 fractions from 10 mice in each group were pooled, resuspended in 320 mM sucrose, 1 mM NaHCO$_3$, and 1 mM PMSF (pH 8.0), and fractionated in sucrose gradients (0.85, 1.0, and 1.2 M) by centrifugation at 82,500g for 2 hours. The synaptosomal plasma membrane between 1.0 and 1.2 M sucrose was incubated with 6 mM tris-HCl (pH 8.0), 160 mM sucrose, 1 mM PMSF, and 0.5% Triton X-100 at 4°C for 15 min, followed by centrifugation at 32,000g for 20 min to obtain PSD fraction (29).
4°C overnight under gentle rotation. The immune complexes were isolated by protein A/G agarose beads, washed six times with RIPA buffer, and boiled in SDS sample buffer (25, 27).

The equal amounts of protein samples were resolved on SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. After blocking in 5% nonfat milk, the membrane was incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) for 1 hour at room temperature. Protein bands were visualized by enhanced chemiluminescence. The following antibodies were used in the current study: rabbit anti-GluN2B, mouse anti–GluN2A, rabbit anti–GluA2, and rabbit anti-ubiquitin (Lys63- or Lys48-specific) antibody from Millipore; mouse anti–GluN1 antibody from BD Pharmingen (San Diego, CA, USA); rabbit anti–Cbl-b and rabbit anti–C-Cbl antibody from Proteintech; rabbit anti–Myc antibody from Abcam (Cambridge, MA, USA); mouse anti–Myc antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti–β-actin, mouse anti-ubiquitin, and mouse anti-phosphotyrosine antibody (clone PY20) from Sigma-Aldrich.

Electrophysiological recordings

To examine the synaptic role of Cbl-b during development, the mice at P5 and P6 were intrathecially injected with lentivirus, and electrophysiological recordings were conducted after 10 to 12 days. To investigate the influence of Cbl-b on synaptic transmission during inflammation pain, the mice at P41 and P42 were injected intradermally with CFA. One day after CFA injection, the adenosine was intrathecally given. Electrophysiological recordings were performed at day 3 after adenoviral injection. To prepare the spinal slices, the roots were stimulated (0.1 Hz, 0.1-ms duration) through a suction electrode. The evoked NMDAR-mediated EPSCs were recorded at +40 mV in the presence of AMPAR antagonist CNQX (10 μM), γ-aminobutyric acid type A receptor antagonist bicuculline (10 μM), and glycine receptor antagonist strychnine (2 μM). The monosynaptic EPSCs were identified on the basis of the constant latency and the absence of conduction failure in response to high-frequency electrical stimulation (20 Hz). The input-output curves were plotted by eliciting synaptic currents at different stimulation intensities (0.1 to 2.0 mA) (30). The series and input resistances were monitored online throughout the experiments. The recordings were abandoned if any of the series and input resistances changed by more than 15%.

Signals were filtered at 2 kHz and digitized at 10 Hz. The experimenters were blinded to the treatment groups.

Statistical analysis

We collected the electrophysiological data from one neuron per mouse. All data were expressed as means ± SEM. The peak amplitudes of EPSCs were analyzed by Clampfit 8.0 software. The weighted decay time constant (τW) of NMDAR-EPSCs was calculated according to the following formula: τW = (1 × τf + I × τs)/(I + I) (I and I, the fast and slow component of NMDAR-EPSCs, respectively; τf and τs the fast and slow decay time constant, respectively). For Western blots, the scanned digital images were quantified by National Institutes of Health ImageJ software. The relative immunoreactive density of each protein was normalized by the averaged density in control group. For immunocytochemical analysis, the fluorescent intensities were measured with Image-Pro Plus 6 software and normalized by the averaged intensity in control cells. We conducted two group comparisons with Mann-Whitney U test. The data across multiple groups were compared by one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test. The repeated measurement and Bonferroni post hoc tests were performed to compare the data between multiple groups occurring over time. The criterion for statistical significance was P < 0.05.

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES


**Funding:** This work was supported by the National Natural Science Foundation of China (number 31771160). **Author contributions:** Z.-Y.Z., Z.G., H.-L.L., X.-T.D., T.-Y.Z., L.Y., J.-J.M., Y.-X.L., X.B., H.-K.C., Z.-W.S., and X.Y. conducted the biochemical experiments. H.-H.B. performed the electrophysiological recordings. Z.-Y.Z. conducted the immunocytochemical experiments. Z.G., T.-Y.Z., and Z.C. performed the behavioral experiments. Z.G., H.-L.L., X.-T.D., and L.Y. performed the immunohistochemical experiments. X.-D.H. contributed to the study design, the data analysis and interpretation, as well as paper writing and revision. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper.

Submitted 22 November 2018
Resubmitted 31 December 2019
Accepted 8 June 2020
Published 30 June 2020
10.1126/scisignal.aaw1519

Ubiquitination and functional modification of GluN2B subunit–containing NMDA receptors by Cbl-b in the spinal cord dorsal horn

Zi-Yang Zhang, Hu-Hu Bai, Zhen Guo, Hu-Ling Li, Xin-Tong Diao, Tian-Yu Zhang, Lin Yao, Juan-Juan Ma, Zheng Cao, Yin-Xia Li, Xue Bai, Hai-Kun Chen, Zhan-Wei Suo, Xian Yang and Xiao-Dong Hu

DOI: 10.1126/scisignal.aaw1519

A painful return to developmental NMDAR activity

Neuronal sensitivity is mediated, in part, by NMDA receptor (NMDAR) signaling, and increased abundance of GluN2B-containing NMDARs in sensory neuronal synapses is associated with enhanced nociception and pain. Using rodents, Zhang et al. found that GluN2B content in the sensory neurons of the spine is progressively restricted during early development and maintained at low abundance by the E3 ubiquitin ligase Cbl-b during adulthood. However, peripheral inflammation induced the dephosphorylation of Cbl-b and impaired its interaction with GluN2B, and the increase in GluN2B abundance enhanced NMDAR activity and neuronal sensitivity to touch. Identifying a way to target this mechanism might be therapeutic in patients suffering from inflammatory peripheral neuropathy.