The Noonan syndrome-associated D61G variant of the protein tyrosine phosphatase SHP2 prevents synaptic down-scaling

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Homeostatic scaling of the synapse, such as synaptic down-scaling, has been proposed to offset deleterious effects induced by sustained synaptic strength enhancement. Proper function and subcellular distribution of Src homology 2 domain-containing nonreceptor protein tyrosine phosphatase (SHP2) are required for synaptic plasticity. However, the role of SHP2 in synaptic down-scaling remains largely unknown. Here, using biochemical assays and cell-imaging techniques, we found that synaptic SHP2 levels are temporally regulated during synaptic down-scaling in cultured hippocampal neurons. Furthermore, we observed that a Noonan syndrome-associated mutation of SHP2, resulting in a D61G substitution, prevents synaptic down-scaling. We further show that this effect is due to an inability of the SHP2-D61G variant to properly disassociate from postsynaptic density protein 95, leading to impaired SHP2 dispersion from synaptic sites after synaptic down-scaling. Our findings reveal a molecular mechanism of the Noonan syndrome-associated genetic variant SHP2-D61G that contributes to deficient synaptic down-scaling.

Synapses, in response to stimuli, undergo changes in synaptic strength in a bidirectional manner to ensure proper synaptic transmission (1–3). However, cumulative synaptic strength enhancement may cause the synapse to be incapable of undergoing synaptic plasticity (4). Therefore, homeostatic scaling of synapses, i.e. synaptic down-scaling, may offset the deleterious effects induced by continuous synaptic strength enhancement (4–7). Synaptic scaling is critical for maintaining optimal neural circuit function and thereby preventing neuronal network dysfunction (8). Accumulating evidence indicates that synaptic scaling is achieved via up-regulation or down-regulation of AMPA receptors (3). AMPA receptors in rodent hippocampi are largely made up of GluA1/GluA2 or GluA2/GluA3 heterodimers (2, 9). Notably, the modulation of surface GluA1-containing AMPA receptors is highly associated with synaptic scaling.

Noonan syndrome affects 1 in 2500 live births and is accompanied by deficits in synaptic plasticity, learning, and memory (10, 11). Gain-of-function mutations in the PTPN11 gene, which encodes a Src homology 2 (SH2) domain-containing nonreceptor protein tyrosine phosphatase (SHP2), are a major genetic contributor in Noonan syndrome, and ~50% of Noonan syndrome patients have functional deficits in SHP2 (11). In Noonan syndrome, the underlying molecular mechanism is the constitutive activation of SHP2. Specifically, the disease-related mutants of SHP2 result in interrupted interaction between the autoinhibitor N-SH2 domain and the catalytic domain of SHP2 (12, 13). Additionally, SHP2 mutants have a higher binding affinity for their binding partners, such as GAB1 (14). In recent years, knock-in mice expressing Noonan syndrome-related mutated SHP2 or with adeno-associated virus (AAV)-mediated expression of Noonan syndrome-associated mutation of SHP2 (i.e. D61G of SHP2) in the hippocampi have been shown to recapitulate the Noonan syndrome-like phenotypes, especially learning and memory deficits (15–18). Previous extensive work elucidated the exquisite molecular mechanisms of memory deficits caused by the constitutive activation of SHP2. However, the functional role of constitutively activated SHP2 in homeostatic plasticity remains largely unknown, especially in synaptic down-scaling elicited by chronic treatment with bicuculline (Bic).

In the current study, we investigated the temporal changes in synaptic SHP2 content during synaptic down-scaling in cultured hippocampal neurons. Moreover, we proposed that the Noonan syndrome-associated mutation SHP2 D61G prevented synaptic down-scaling via elevated association with PSD95, leading to impaired SHP2 dispersion from postsynaptic sites during synaptic down-scaling.

Results

Time course of synaptic SHP2 during synaptic down-scaling

Our previous study indicated that TTX-induced synaptic up-scaling yields up-regulation of synaptic SHP2 (19). However, the distribution of Shp2 during synaptic down-scaling by chronic Bic treatment remains largely unknown. Synaptic down-scaling can be achieved in cultured neurons by prolonged elevation of neuronal activity elicited by Bic. Thus, we sought out to explore the synaptic content of SHP2 during synaptic down-scaling via a battery of analyses (Fig. 1A). We measured miniature
excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons to examine the time course of synaptic down-scaling. We observed a gradual decrease in mEPSC amplitudes from 12 to 48 h of Bic treatment (Fig. 1, B and C). Notably, only the amplitude of mEPSCs in 24- or 48-h Bic-treated neurons was significantly different from that of the control neurons (control, 18.9 ± 3.1 pA; Bic for 12 h, 17.9 ± 5.3 pA; Bic for 24 h, 11.3 ± 2.2 pA; Bic for 48 h, 10.8 ± 2.3 pA). Next, we measured the colocalization level of SHP2 and PSD95 by immunocytochemistry in cultured neurons treated
with Bic, at different time points. Mander’s coefficient analysis demonstrated that 24 h or 48 h of Bic treatment elicited reduced levels of colocalization of SHP2 and PSD95 in dendrites (control, 0.46 ± 0.07; Bic for 12 h, 0.40 ± 0.05; Bic for 24 h, 0.33 ± 0.07; Bic for 48 h, 0.31 ± 0.06) (Fig. 1, D and E), which matched the time course of mEPSC amplitudes. However, the intensities of PSD95 (control, 1.00 ± 0.17; Bic for 12 h, 1.06 ± 0.10; Bic for 24 h, 0.98 ± 0.10; Bic for 48 h, 0.93 ± 0.10) and SHP2 (control, 1.00 ± 0.18; Bic for 12 h, 1.17 ± 0.19; Bic for 24 h, 1.07 ± 0.18; Bic for 48 h, 1.04 ± 0.16) in dendrites were not altered during synaptic down-scaling (Fig. 1F). To support the results from immunocytochemistry, we evaluated the SHP2 content in postsynaptic density (PSD) fractions during synaptic down-scaling by Western blotting analysis. SHP2 levels in PSD fractions were decreased in all Bic-treated groups, relative to control, excluding those in the 12-h Bic-treated group, which were only marginally and not significantly reduced, compared with those in control (Bic for 12 h, 0.88 ± 0.06; Bic for 24 h, 0.47 ± 0.08; Bic for 48 h, 0.38 ± 0.13) (Fig. 1, G and H). The time scale by which the GluA1 level is down-regulated in PSD fractions by chronic Bic treatment correlates with the reduction of the SHP2 levels in PSD fractions. Specifically, we found an overt reduction of GluA1 only in 24-h or 48-h Bic-treated group (Bic for 12 h, 0.95 ± 0.13; Bic for 24 h, 0.47 ± 0.09; Bic for 48 h, 0.39 ± 0.10) (Fig. 1, G and H). A previous study indicated that SHP2 associates with PSD95 (20). Hence, we wondered whether SHP2 dissociated from PSD95 during synaptic down-scaling. Utilizing coimmunoprecipitation and Western blotting, we found that coimmunoprecipitation of SHP2 with PSD95 was reduced in the 24-h and 48-h Bic-treated groups, while the 12-h Bic-treated group showed a slight and not significant decrease in SHP2 coimmunoprecipitated by PSD95 antibody (Bic for 12 h, 0.78 ± 0.08; Bic for 24 h, 0.45 ± 0.14; Bic for 48 h, 0.42 ± 0.14) (Fig. 1, I and J). Taken together, these data show that there is a time course-dependent reduction of synaptic SHP2 at 24 h and 48 h during synaptic down-scaling, which is likely due to decreased association of SHP2 and PSD95.

Synaptic down-scaling is prevented in SHP2 D61G-expressing neurons

A prominent role for SHP2 in regulating synaptic up-scaling was proposed in our previous study (19). Given that synaptic up-scaling requires proper SHP2 function, we hypothesized that the Noonan syndrome-related mutation of SHP2 at D61G, which causes hyperactivation of SHP2, may prevent synaptic down-scaling. To test this hypothesis, we infected cultured hippocampal neurons at DIV 7 with AAVs expressing either WT SHP2 as control or SHP2 with the D61G mutation (Fig. 2A). Seven days postinfection, intense GFP signals were detected in both AAV-SHP2 WT-expressing or AAV-SHP2 D61G-expressing neurons under confocal microscopy, suggesting comparable ectopic expression levels of SHP2 WT and SHP2 D61G in cultured hippocampal neurons (D61G, 1.08 ± 0.17, compared with WT) (Fig. 2, B and C). Subsequently, we assessed the effects of overexpression of SHP2 WT and SHP2 D61G in the hippocampal neurons. The surface GluA1 level but not total GluA1 level was increased in SHP2 D61G-expressing neurons, compared with the uninfected or SHP2 WT-expressing neurons, while the surface GluA1 levels and total GluA1 levels were similar between uninfected and SHP2 WT-expressing neurons (Fig. S1, A and B). These results suggest hyperactivity of SHP2 that leads to elevated surface GluA1 levels in the neurons due to overexpression of SHP2 D61G. At DIV 14, Bic was applied to the AAV-infected neurons for 48 h to induce synaptic down-scaling. mEPSC recordings revealed that cultured neurons infected with AAV-SHP2 WT showed a robust decrease in amplitude after 48 h of Bic treatment. Conversely, this reduction in amplitude was not observed in cultured neurons infected with the AAV-SHP2 D61G after 48 h of Bic treatment (Fig. 2, D and E). Notably, AAV-SHP2 D61G yielded an increase in the amplitude of neurons with or without Bic treatment, compared with AAV-SHP2 WT-expressing cultured neurons (Fig. 2, F and G) (WT: control, 19.8 ± 2.2 pA; Bic for 48 h, 14.2 ± 1.5 pA; D61G: control, 23.4 ± 1.9 pA; Bic for 48 h, 23.1 ± 1.8 pA). Although a slight but not significant increase of mEPSC frequency was detected in AAV-SHP2 D61G-expressing neurons, relative to AAV-SHP2 WT-expressing neurons, the mEPSC frequency was not significantly changed in the four groups (Fig. 2F) (WT: control, 2.84 ± 0.69 Hz; Bic for 48 h, 2.87 ± 0.67 Hz; D61G: control, 3.36 ± 0.85 Hz; Bic for 48 h, 3.38 ± 0.75 Hz). Additionally, we measured the surface intensity of GluA1 in either AAV-SHP2 WT-expressing or SHP2 D61G-expressing neurons with or without Bic treatment. Similarly, 48 h of Bic treatment indeed decreased the surface intensity of GluA1 in AAV-SHP2 WT-expressing neurons, while the surface GluA1 level remained unchanged in SHP2 D61G-expressing neurons after 48 h of Bic treatment (WT: control, 1.00 ± 0.17; Bic for 48 h, 0.68 ± 0.14; D61G: control, 1.24 ± 0.16; Bic for 48 h, 1.24 ± 0.16) (Fig. 2, G and H). Consistent with the mEPSC recordings, AAV-SHP2 D61G-expressing neurons exhibited higher basal surface GluA1 levels, which might impede synaptic down-scaling in AAV-SHP2 D61G-expressing neurons. We hypothesized that enzymatic hyperfunction of SHP2 D61G may lead to the deficient down-scaling, which is likely due to decreased association of SHP2 and PSD95.

Figure 1. Time course of synaptic SHP2 content during Bic-mediated synaptic down-scaling. A schematic drawing showing the experimental design. B, representative traces of mEPSC recordings from cultured hippocampal neurons with or without Bic treatment for the indicated time. C, bars represent the means of the quantified data for the mEPSC recordings in B. The dots within the bars indicate the numbers of neurons from three independent cultures (n = 9 cells). One-way ANOVA with Bonferroni post hoc analysis. D, confocal images showing of SHP2 and PSD95 in cultured hippocampal neurons treated as in A. Scale bar, 10 μm. E, Mander’s coefficient of SHP2 (red) colocalized with PSD95 (green). F, summary data of relative intensity of PSD95 (left) and SHP2 (right) in D. The dots within the bars indicate the numbers of neurons from three independent cultures (n = 12 neurons). One-way ANOVA with Bonferroni post hoc analysis. G, protein levels of GluA1 and SHP2 in total cell lysates and PSD fractions from cultured hippocampal neurons treated as in A were assessed by Western blotting and densitometric analyses. H, quantification of protein levels in total cell lysates (left) or PSD fractions (right) (n = 4 independent experiments from four cultures). Bic-treated groups were normalized to the control group. One-way ANOVA with Bonferroni post hoc analysis. I, protein lysates of hippocampal neurons, treated as in A, subjected to immunoprecipitation (IP) with the anti-PSD95 antibody. Input and immunoprecipitated samples were assessed by Western blotting and densitometric analyses. J, summary data of total SHP2 level (left) and PSD95-associated SHP2 level (right) in H (n = 4 independent experiments from four cultures). All Bic-treated groups were normalized to the control group. One-way ANOVA with Bonferroni post hoc analysis. Error bars depict S.D. #, p ≤ 0.001; n.s., no significance.
synaptic down-scaling. To test this, we took advantage of a catalytically inactive SHP2 mutant (C459S). Hence, we generated a SHP2 D61G/C459S double mutant vector. Subsequently, we transfected SHP2 WT or SHP2 D61G/C459S vector alone with the EGFP-N1 vector into hippocampal neurons at DIV 7. The effect of chronic Bic treatment on the surface level of GluA1 in GFP-positive hippocampal neurons (DIV 16) was assessed. Strikingly, SHP2 WT-expressing neurons showed decreased surface GluA1 levels after Bic treatment, whereas the decrease of surface GluA1 was not observed in SHP2 D61G/C459S-expressing neurons after 48 h of Bic treatment (Fig. S2, A and B) (WT: control, 1.00 ± 0.14; Bic for 48 h, 0.64 ± 0.10; D61G/C459S: control, 1.08 ± 0.16; Bic for 48 h, 0.97 ± 0.18). These results suggest that hyperactivity of SHP2 D61G contributes to the elevation of surface GluA1 levels but not the disruption of synaptic down-scaling. Collectively, these data suggest that the Noonan syndrome-associated mutation of SHP2 at D61G results in impaired synaptic down-scaling.

**Impaired dispersion and enhanced interaction with PSD95 of SHP2 D61G after synaptic down-scaling**

Given that synaptic content of SHP2 decreased after synaptic down-scaling in neurons and SHP2 D61G/C459S failed to restore synaptic down-scaling, we next asked whether the loss of synaptic down-scaling in SHP2 D61G-expressing neurons was due to impaired dispersion of SHP2 at postsynaptic sites. To determine this, the level of SHP2 and PSD95 colocalization was evaluated in AAV-SHP2 WT-expressing or AAV-SHP2 D61G-expressing neurons with or without 48 h of Bic treatment. Interestingly, a lower level of SHP2 colocalized with PSD95 in AAV-SHP2 WT-expressing neurons was found following 48 h of Bic treatment. Conversely, the level of SHP2 and PSD95 colocalization was unaltered in AAV-SHP2 D61G-expressing neurons with or without 48 h of Bic treatment (WT: control, 0.44 ± 0.07; Bic for 48 h, 0.33 ± 0.07; D61G: control, 0.53 ± 0.06; Bic for 48 h, 0.53 ± 0.05) (Fig. 3, A and B). However, the intensity of PSD95 (WT: control, 1.00 ± 0.20; Bic for
48 h: 0.94 ± 0.09; D61G: control, 1.02 ± 0.17; Bic for 48 h: 0.95 ± 0.11) or SHP2 (WT: control, 1.00 ± 0.18; Bic for 48 h, 1.09 ± 0.19; D61G: control, 1.04 ± 0.14; Bic for 48 h: 1.10 ± 0.18) in dendrites was not significantly changed with or without 48 h of Bic treatment in AAV-SHP2 WT-expressing or AAV-SHP2 D61G-expressing neurons (Fig. 3C). To further measure the association of SHP2 and PSD95, SHP2 was coimmunoprecipitated by PSD95 antibody in both AAV-SHP2 WT-expressing and AAV-SHP2 D61G-expressing neurons with or without 48 h of Bic treatment. As expected, AAV-SHP2 WT-expressing neurons exhibited decreased SHP2 coimmunoprecipitated by the PSD95 antibody after 48 h of Bic treatment. However, the level of PSD95-associated SHP2 was unchanged in AAV-SHP2 D61G-expressing neurons with or without 48 h of Bic treatment (WT: Bic for 48 h, 0.65 ± 0.07 of WT control; D61G: control, 1.40 ± 0.14 of WT control; Bic for 48 h, 1.35 ± 0.16 of WT control) (Fig. 3, D and E). These results suggest that impaired dispersion of SHP2 D61G following synaptic down-scaling may be the molecular mechanism leading to deficient synaptic down-scaling in Noonan syndrome.

**Discussion**

SHP2 was identified as a protein associated with PSD95 and localized at excitatory synapses (20, 21). The present study, to our knowledge, is the first to discover that the Noonan syndrome.
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syndrome-associated mutation of SHP2 at D61G prevented synaptic down-scaling, likely through enhanced interaction with PSD95. During chronic Bic treatment, SHP2 WT underwent dispersion from postsynaptic sites, while SHP2 D61G remained at postsynaptic sites (Fig. 3F). However, we cannot rule out other possible disruptive mechanisms of SHP2 D61G in hippocampal neurons, i.e. structural dysregulation of SHP2 D61G or overexpression of SHP2.

In the present study, we show that SHP2 associates with PSD95 in hippocampal neurons. However, whether this association of SHP2 with PSD95 is direct or indirect needs further investigation. Upon chronic Bic treatment, SHP2 or PSD95 may undergo posttranslational modifications and conformational changes, and these alterations can lead to dissociation of SHP2 from PSD95. There are other possibilities, for example, translocation of SHP2 from dendritic spines to the nucleus (22, 23).

Of note, we failed to detect any significant change of mEPSC frequency in AAV-SHP2 D61G-expressing neurons, compared with AAV-SHP2 WT-expressing neurons, which would seem to be discrepant from the results obtained in a previous study (16). One possible reason for the difference is that our study used hippocampal neuron cultures and the aforementioned study used acute hippocampal slices with AAV infection (16). However, a recent report shows unchanged mEPSC frequency or amplitude in heterozygous SHP2 D61G knock-in mice, compared with its WT littermates (24). Importantly, a recent study revealed that transient overexpression of SHP2 D61G in cultured hippocampal neurons at different developmental stages differentially affected the degree of surface expression of GluA1 (25). These inconsistencies need to be further resolved in the future. Nonetheless, regardless of the disposition of SHP2 D61G on surface expression of GluA1, all evidence suggests that SHP2 D61G seems to limit the ability of neurons to undergo various forms of plasticity. Albeit with reduced GluA1 and SHP2 contents in postsynaptic sites, we did not observe any obvious reduction of PSD95, which is in accordance with a previous study showing a comparable level of PSD95 in hippocampal neurons after 48 h of Bic treatment (26). Interestingly, it is worth noting that PSD95 is decreased in cortical neuron cultures after Bic treatment (27), suggesting differential regulation of PSD95 in hippocampal neurons and cortical neurons after synaptic down-scaling.

Synaptic scaling, which triggers compensatory synaptic changes, is a critical molecular mechanism needed to maintain neuronal firing within an optimal range and proper neural circuit function (4). During the past decade, numerous studies have convincingly demonstrated the critical role of SHP2 in synaptic plasticity and illustrated how mutated SHP2 causes learning and memory deficits in Noonan syndrome (15–18, 21). Although the crucial role of SHP2 in synaptic plasticity has been well established, SHP2 regulation and function in synaptic scaling are largely unknown. Herein, we indicate that one of the Noonan syndrome-associated mutations, SHP2 D61G, disrupted synaptic down-scaling. Additionally, several gain-of-function mutations of SHP2, such as E76K and N308D, have been found in Noonan syndrome (28). Therefore, the functional role of these other Noonan syndrome-associated mutations of SHP2 in synaptic scaling should be explored in the future.

Our results indicate that SHP2 D61G-expressing neurons prevent synaptic down-scaling. With advances in stem cell technology, the impact of SHP2 D61G on synaptic down-scaling should be determined in the future in induced pluripotent stem cell-derived neurons from Noonan syndrome patients. This would allow for the evaluation of synaptic down-scaling in induced pluripotent stem cell-derived neurons, to directly study the molecular basis of memory deficits shown in Noonan syndrome patients.

Experimental procedures

DNA constructs and viral packaging

AAVs, both AAV-CMV-GFP-SHP2 WT and AAV-CMV-GFP-SHP2 D61G, were designed and constructed by standard methods and were packaged by Obio Technology. cDNA encoding full-length SHP2 was a gift from Dr. Yuehai Ke, Zhejiang University. The SHP2 D61G mutant and the SHP2 D61G/C459S double mutant were generated by standard site-directed mutagenesis using the QuikChange mutagenesis system, as described previously. The EGFP-N1 vector was from Clontech. All DNA constructs were verified by DNA sequencing before use.

Animal care

All animal protocols used were approved by the Animal Advisory Committee at Hangzhou Normal University (Approval SYXY (Zhejiang) 2016-0014) and followed NIH guidelines for the care and use of laboratory animals. C57BL/6j mice were used and housed individually under standard conditions of temperature and humidity and a 12 h light/dark cycle, with free access to food and water. We made maximal efforts to minimize animal numbers used and to reduce animal suffering.

Hippocampal neuron cultures, transfection, and synaptic down-scaling induction by Bic

Hippocampal primary neuronal cultures were harvested from newborn (P1–P2) C57BL/6j mice of either sex as described (19, 21). Hippocampal tissue was quickly dissected in ice-cold Hanks’ balanced salt solution (Gibco) before dissociation in 0.25% trypsin for 15–18 min at 37 °C. Dissociated neurons were plated on poly-1-lysine (Sigma)-coated 35-mm dishes (Corning) for biochemical experiments, 12-mm coverslips (Deckglass) for cell-imagining experiments, and 6-mm coverslips (Deckglass) for electrophysiological experiments, in primary neurobasal medium (Gibco) containing 10% horse serum at 37 °C under 5% CO2. Two hours later, cells were maintained in neurobasal medium containing 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mm GlutaMax supplemented with 2% B-27 supplement (Gibco). Subsequently, half of the culture medium was replaced every 4 d. At DIV 4, cytosine arabinoside was added to the culture medium at a final concentration of 2.5 μM, to inhibit the growth of glia.

SHP2 WT or SHP2 D61G/C459S double mutant was cotransfected with EGFP-N1, at a mass ratio of 10:1, into hippocampal
neurons at DIV 7 using the CalPhos mammalian transfection kit (Clontech). The protocol for synaptic down-scaling was adopted from a previous publication with minor modifications (29). Hippocampal neurons were infected with AAVs (AAV-CMV-GFP-SHP2 WT or AAV-CMV-GFP-SHP2 D61G) at DIV 7. At DIV 14, neurons were incubated with Bic at a final concentration of 20 μM for the indicated time (12 h, 24 h, or 48 h). Neurons treated with DMSO were considered the control group.

**mEPSC recordings**

mEPSCs were measured in cultured hippocampal neurons at DIV 16 as described previously (30). GFP-positive neurons were voltage clamped with the whole-cell patch technique during recording at room temperature. The bath solution (pH 7.4, 290–310 mosm) contained 119 mM NaCl, 2.5 mM KCl, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 11 mM D-glucose, 2.5 mM CaCl₂, and 1.3 mM MgSO₄, with 100 μM μrotoxin and 1 μM TTX. Glass patch pipettes with resistances of 3–5 MΩ were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM NaATP, 0.4 mM NaGTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA (pH 7.4, 290–310 mosm). All reagents were purchased from Merck (Sigma) unless stated otherwise. Electrical signals were acquired at 10 kHz with a MultiClamp 700B amplifier using pClamp10 software (Molecular Devices). Cells were held at −60 mV, and mEPSC measurements were collected for 7–10 min. mEPSC data were analyzed using Mini Analysis software (Synaptosoft, Decatur, GA, USA). The filter frequency for the acquired electrophysiological data was 1.5 Hz, and the threshold criterion was 5 pA for analyzing events. Data are presented as the mean ± S.D., where n represents the number of cells.

**Immunocytochemistry and imaging data analysis**

For surface GluA1 labeling, neurons were washed twice with 37 °C PBS, fixed for 5 min at room temperature in PBS containing 4% paraformaldehyde, blocked for 30 min with 5% BSA in PBS, and then incubated for 15 min with mouse anti-GluA1 (1:50, MAB2263; Millipore) in PBS at 37 °C. After extensive washing, the neurons were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 5% BSA, followed by labeling with rabbit anti-mitogen-activated protein 2 (MAP2) antibody (1:1000, no. 4542; CST). For staining of endogenous SHP2, PSD95, or MAP2, neurons were fixed for 10 min in PBS containing 4% paraformaldehyde at room temperature and then permeabilized as well as blocked for 30 min in PBS buffer containing 0.2% Triton X-100 and 5% BSA. SHP2 was labeled with mouse anti-SHP2 (1:50, no. 610621; BD), PSD95 was labeled with rabbit anti-PSD95 (1:100, no. 3409S; CST), and MAP2 was labeled with rabbit anti-MAP2 (1:1000, no. 4542, CST) primary antibody for 1 h at room temperature. Neurons were extensively washed and incubated with the respective secondary antibody for 1 h at room temperature or overnight at 4 °C. For colocalization experiments, Alexa 488-conjugated donkey anti-mouse IgG (1:500, no. A21202; Invitrogen) and Alexa 546-conjugated donkey anti-rabbit IgG (1:500, no. A10040; Invitrogen) secondary antibodies were used. For AAV-mediated SHP2 WT- or SHP2 D61G-expressing neurons, SHP2 or surface GluA1 was labeled with Alexa 546-conjugated goat anti-mouse IgG secondary antibody (1:500, no. A11030; Invitrogen) and PSD95 or MAP2 was labeled with Alexa 633-conjugated goat anti-rabbit IgG secondary antibody (1:500, no. A21082; Invitrogen). Cells were imaged on a confocal microscope (LSM 710; Zeiss). The colocalization level was analyzed using ImageJ, and results are presented as mean ± S.D. For surface AMPA receptor, SHP2, or PSD95 intensity, the integrated intensities of individual puncta of endogenous surface GluA1, SHP2, or PSD95 on the dendrite were measured. Experiments were repeated at least three times in independent cultures.

**Obtainment of PSD fraction**

Obtainment of the PSD fraction was performed according to previously reported methods with minor modifications (19, 31). Cultured hippocampal neurons were homogenized in ice-cold buffer (pH 7.4) containing 320 mM sucrose and 10 mM HEPES and were centrifuged at 1000 × g for 4 min. The supernatant was subsequently centrifuged twice at 12,000 × g for 20 min to obtain the pellet as the crude membrane fraction. Afterward, the pellet was rinsed with buffer (pH 7.4) containing 4 mM HEPES and 1 mM EDTA, and this was repeated once by precipitation at 12,000 × g for another 20 min. The pellet from centrifugation was dissolved in preprimary lysis buffer (pH 7.2) containing 20 mM HEPES, 100 mM NaCl, and 0.5% Triton X-100, incubated for 15 min, and subjected to centrifugation at 12,000 × g for 20 min. The pellet was further lysed for 1 h in final lysis buffer (pH 7.2) containing 20 mM HEPES, 0.15 mM NaCl, 1 mM DTT, 1% Triton X-100, 1% deoxycholic acid, and 1% SDS and was subjected to centrifugation at 10,000 × g for 15 min. The supernatant was regarded as the PSD fraction and subjected to further analysis. All buffers were supplemented with protease and phosphatase inhibitors. All of the procedures mentioned above were performed at 4 °C.

**Coimmunoprecipitation**

Coimmunoprecipitation was carried out as described previously (31, 32). Hippocampal cultures were lysed with RIPA buffer (Beyotime, Shanghai, China) for 1 h at 4 °C. After two ultrasonication steps for 8 s each time, the extracted lysate was centrifuged at 12,000 × g for 10 min at 4 °C and the supernatant was collected and adjusted to 1 μg/μl for input. The PSD95 antibody (2 μg) was added to another 800 μl of the sample and incubated overnight at 4 °C. Next, 30 μl of solubilized protein A-Sepharose beads were added to the sample and incubated for 4 h. The sample was rinsed three times with lysis buffer containing 500 mM NaCl, to remove nonspecific interactions. After centrifugation, protein A-Sepharose beads were incubated with 2× sample buffer at 100 °C for 10 min. Finally, the sample was centrifuged at 16,000 × g for 1 min, and the supernatant was used for Western blotting.

**Biotinylation assay**

Hippocampal neurons were infected with AAVs at DIV 7. At DIV 14, the neurons were collected and the biotinylation assay was performed according to our previous method (31).
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Immunoblotting

The process of preparing cultured neurons for immunoblotting is the same as described in our previous publications (21, 31). The samples were quantified using a BCA protein assay kit (Thermo Scientific). Samples containing equal protein amounts were loaded into each lane, separated on 8% or 10% SDS-PAGE gels, and then transferred onto nitrocellulose membranes (Whatman, GE Healthcare Life Sciences). The blots were probed with corresponding primary antibodies and incubated with HRP-conjugated secondary antibodies (Thermo Scientific). The blots were developed with ECL reagent (SuperSignal West Dura, no. 34076; Thermo Scientific). Chemiluminescence was detected by photographic film. Densitometry of the relevant bands was performed using Quantity One software (Bio-Rad).

Antibodies and reagents

The primary antibodies used for immunoblotting were rabbit anti-SHP2 (1:4000, no. SC-280; Santa Cruz), rabbit anti-GFP (1:1000, no. ab183734; Abcam), mouse anti-GluA1 (1:3000, no. MAB2263; Millipore), mouse anti-PSD95 (1:3000, no. ab2723; Abcam), and mouse anti-β-actin (1:10,000, no. A5316; Sigma-Aldrich). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, no. 31420; Pierce, Thermo Scientific) and HRP-conjugated goat anti-mouse IgG secondary antibody (1:10,000, no. 31460; Pierce, Thermo Scientific). TTX (no. 1078) and Bic (no. 2503) were acquired from Tocris.

Statistics

All data are represented as mean ± S.D. The comparisons of two groups were done by the two-tailed Student t test. Multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. A p value of <0.05 was considered significant. All tests were done using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA).

Data availability

All data are contained within this article and supporting information.

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Abbreviations—The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SH2, Src homology 2 domain-containing nonreceptor protein tyrosine phosphatase; Bic, bicuculline; TTX, tetrodotoxin; DIV, day in vitro; AAV, adeno-associated virus; PSD, postsynaptic density; PSD95, postsynaptic density protein 95; MAP2, mitogen-activated protein 2; ANOVA, analysis of variance; mEPSC, miniature excitatory postsynaptic current; EGFP, enhanced green fluorescent protein.

References


