

Research article

Noonan syndrome-associated SHP2 mutation differentially modulates the expression of postsynaptic receptors according to developmental maturation



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HIGHLIGHTS

- A Noonan syndrome-associated mutation in SHP2 increases NMDA receptor expression in premature neurons.
- This mutation increases the size of AMPA receptor clusters in early maturing neurons.
- The SHP2 mutation also increases both the size and the number of AMPA receptor clusters in mature neurons.
- The altered expressions of glutamate receptors by mutant SHP2 can be reversed by inhibiting the MAPK signaling pathway.

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ABSTRACT

Glutamate is the major excitatory neurotransmitter in the central nervous system, and related signaling involves both AMPA and NMDA subtype receptors. The expression of glutamate receptors is dynamically regulated during development. Recent studies showed that the dysregulation of glutamate receptor expression and function is associated with neurodevelopmental disorders including intellectual disability. Previously, a Noonan syndrome (NS)-associated SHP2 mutation (SHP2^{D61G}) was shown to increase the synaptic delivery of AMPA receptor, subsequently impairing synaptic plasticity and learning in adult mice. However, how the mutant SHP2 affects glutamate receptor expression during development is not known. Here, we found that the SHP2^{D61G} differentially regulates the expression of AMPA and NMDA receptors depending on the stage of neuronal maturation. In cultured neurons (immature stage; DIV 6), overexpression of SHP2^{D61G} significantly increased the average size and the number of NMDA receptor-containing particles, but not those with AMPA receptors. In early matured neurons (DIV 12), SHP2^{D61G} significantly increased only the average size of AMPA receptor particles, and subsequently increased their number in matured neurons (DIV 18). Importantly, all the changes described above for SHP2^{D61G} neurons were reversed by inhibiting MAPK. These data demonstrate that the increased activation of MAPK signaling pathway by SHP2^{D61G} could deregulate the surface expression of synaptic receptors during neuronal development, which likely contributes to cognitive impairments in NS patients.

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1. Introduction

Excitatory synaptic transmission in the central nervous system is mainly mediated by two subtypes of glutamate receptors: AMPA

and NMDA receptors. The composition of glutamate receptors at the synapse is dynamically regulated both in developing and in the adult brain. In early developmental stages, NMDA receptors appear first and AMPA receptor levels gradually increase [1]. The composition of NMDA receptors also changes during development. While the GluN2B subtype is highly expressed in early developmental stages, its expression is significantly decreased in the adult [2]. Activity-dependent changes in the surface expression of AMPA

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receptors are critically involved in synaptic plasticity in multiple brain regions [3,4].

Receptor expression and trafficking can be regulated by multiple signaling pathways. The Ras-MAPK pathway modulates the transport of AMPA receptors to postsynaptic sites [5]. Accordingly, deregulation of Ras-MAPK signaling disrupts synaptic AMPA receptor composition and subsequently impairs synaptic plasticity. Synaptic Ras GTPase-activating protein 1 (SYNGAP1) haploinsufficiency increases Ras activation and enhances AMPA receptor-mediated currents in the developing brain [6]. Previously, we showed that a Noonan syndrome (NS)-associated SHP2 mutation (SHP2^{D61G}) enhances both Ras-MAPK signaling and the expression of postsynaptic AMPA receptors in fully matured cultured neurons [day *in vitro* (DIV) 21] and in the adult hippocampus [7]. NS is a relatively common developmental disorder associated with cardiac defects, growth delay, facial abnormalities and learning disabilities [8,9]. Although NS-associated mutations can have a large impact on neural development [10], and SHP2 is expressed in both mitotic and postmitotic neurons in the developing brain [11], how NS-associated mutations affect glutamate receptor expression in early developmental states is not known. In this study, we investigated the effect of overexpressing SHP2^{D61G} on the cell surface expression of glutamate subtypes at different stages of development *in vitro* (DIV 6, 12, and 18). We found that mutant SHP2 increases the level of NMDA receptors at the neuronal membrane surface at early stage, whereas it increases the surface level of AMPA receptors at later stage. Importantly, all these changes in composition of glutamate receptors could be reversed by inhibiting MAPK signaling. Thus, our data imply that NS-associated SHP2 mutations disrupt the expression dynamics of glutamate receptor subtypes during neuronal maturation.

2. Materials and methods

2.1. Neuron culture

Dissociated hippocampal neuron cultures were prepared from 1-day-old rat pups, plated at ~10,000 neurons/coverslip, and maintained with Neurobasal-A medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen) before use as previously reported [12].

2.2. Sindbis virus

Construction and packaging of the Sindbis virus expressing SHP2^{D61G} has been previously described [7]. After the addition of viral particles to media, cultures were incubated for 11 h for transgene expression.

2.3. Immunohistochemistry and confocal imaging

Immunohistochemistry has been performed as previously described [13]. Briefly, cultured neurons were fixed with 4% paraformaldehyde solution and subjected to immunostaining without permeabilization. The following antibodies were used: polyclonal anti-GluN1 (extracellular) antibody (#AGC-001, alomone labs, Jerusalem, Israel), polyclonal anti-GluA1 (extracellular) antibody (#AGC-004, alomone labs), CyTM3-conjugated goat anti-rabbit IgG antibody (#111-165-003, Jackson ImmunoResearch Lab, West Grove, PA). Images were acquired by using confocal microscope (Zeiss 710, Carl Zeiss, Oberkochen, Germany) and analyzed by using NIH ImageJ software (ver. 1.47v). All procedures of image acquisition and analysis were performed by experimenters blind to experimental condition.

2.4. Drug treatment

Cultured neurons were infected with the Sindbis virus expressing SHP2^{D61G} at the day indicated, and incubated for an additional 11 hours followed by 10 μ M PD98059 (Tocris, Avonmouth, UK) or dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO) treatment for 1 h. Then, cultures were immediately fixed and subjected to immunostaining.

2.5. Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 software (La Jolla, CA). Unpaired *t*-tests were used to determine statistical significance between groups. Two-way analyses of variance (ANOVA) were used to determine the effect of drug treatment. Error bars indicate the standard error (SEM).

3. Results

3.1. Mutant SHP2 overexpression at DIV 6 increases the surface expression of NMDA receptors

We examined the effect of expressing SHP2^{D61G} on the surface expression of glutamate receptor subtypes in cultured hippocampal neurons at different stages of maturation: DIV 6 for immature, DIV 12 for early mature, and DIV 18 for fully mature neurons [14,15]. We overexpressed SHP2^{D61G} in cultured hippocampal neurons DIV 6 by using a bicistronic Sindbis viral vector co-expressing enhanced green fluorescent protein (EGFP) as a reporter, and analyzed the surface expression of glutamate receptors using the antibodies that detect the extracellular N-terminus for GluN1 and GluA1 for NMDA and AMPA receptors, respectively. The overexpression of SHP2^{D61G} did not affect gross morphology of neurons compared to that of GFP expressing control neurons. In addition, SHP2^{D61G} did not affect the expression of other non-target genes such as Rab-4 or actin (Supplementary Fig. 1). Surprisingly, the surface expression of GluN1 was significantly increased by SHP2^{D61G} expression. Both the size and the number GluN1-containing clusters were significantly increased in SHP2^{D61G}-expressing neurons compared to EGFP-expressing control neurons (Fig. 1A and C; GluN1 particle size: SHP2^{D61G}, $0.32 \pm 0.0 \mu\text{m}^2$, $n=15$ neurons, $1450 \mu\text{m}$ of dendrites; EGFP, $0.18 \pm 0.0 \mu\text{m}^2$, $n=20$ neurons, $1803 \mu\text{m}$ of dendrites; unpaired *t*-test, ** $p < 0.01$; GluN1 particle number per $10 \mu\text{m}$: SHP2^{D61G}, 14.62 ± 1.0 , $n=15$ neurons, $1450 \mu\text{m}$ of dendrites; EGFP, 11.31 ± 0.8 , $n=20$ neurons, $1803 \mu\text{m}$ of dendrites; unpaired *t*-test, * $p < 0.05$). In contrast, SHP2^{D61G} overexpression did not affect the surface expression of GluA1 at DIV 6 (Fig. 1B and D; GluA1 particle size: SHP2^{D61G}, $0.12 \pm 0.1 \mu\text{m}^2$, $n=21$ neurons, $1894 \mu\text{m}$ of dendrites; EGFP, $0.11 \pm 0.0 \mu\text{m}^2$, $n=16$ neurons, $1394 \mu\text{m}$ of dendrites; unpaired *t*-test, $p=0.768$; GluA1 particle number per $10 \mu\text{m}$: SHP2^{D61G}, 8.66 ± 0.8 , $n=21$ neurons, $1894 \mu\text{m}$ of dendrites; EGFP, 10.97 ± 1.0 , $n=16$ neurons, $1394 \mu\text{m}$ of dendrites; unpaired *t*-test, $p=0.069$). Importantly, the total expression level of GluN1 was not affected by the expression of SHP2^{D61G} (Supplementary Fig. 1).

The majority of the NS-associated mutations in SHP2, including SHP2^{D61G}, are gain-of-function mutations that enhance Ras-MAPK activation [7,16]. Consistently, we found that the SHP2^{D61G}-expressing neurons treated with MAPK/Erk kinase (MEK) inhibitor PD98059 (10 μ M, 1 h) showed unchanged number and size of surface GluN1 particles compared to EGFP controls (Fig. 1E, GluN1 particle size: SHP2^{D61G}, $0.19 \pm 0.0 \mu\text{m}^2$, $n=25$ neurons, $2378 \mu\text{m}$ of dendrites; EGFP, $0.14 \pm 0.0 \mu\text{m}^2$, $n=24$ neurons, $2207 \mu\text{m}$ of dendrites; SHP2^{D61G} + PD98059, $0.09 \pm 0.0 \mu\text{m}^2$, $n=21$ neurons, $2012 \mu\text{m}$ of dendrites; EGFP + PD98059, $0.12 \pm 0.0 \mu\text{m}^2$,

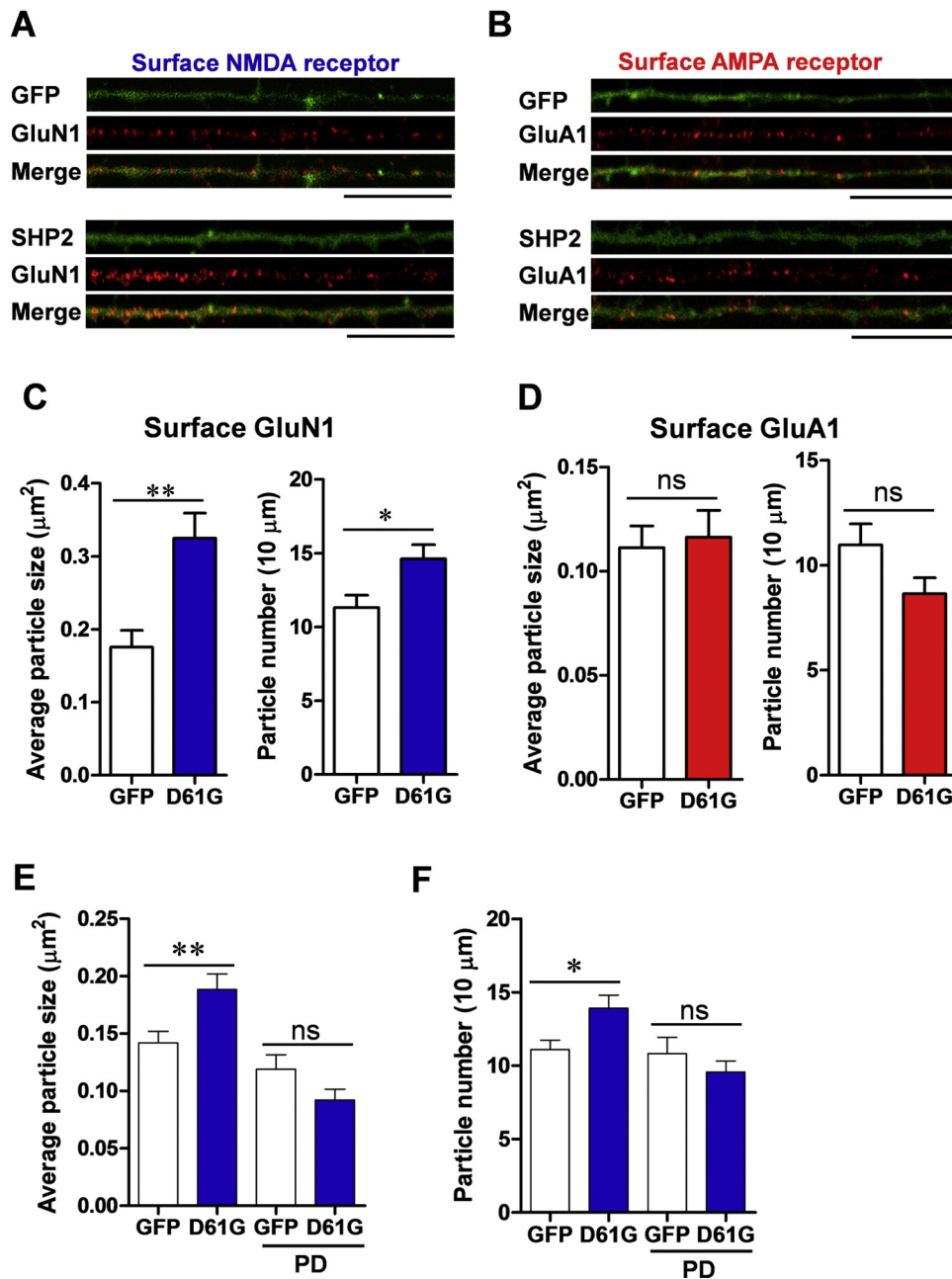


Fig. 1. SHP2^{D61G} overexpression at DIV6 increases surface NMDA receptor expression. (A,B) Representative images of surface GluN1 (A) or GluA1 (B) receptor immunostaining. GFP alone (upper) or SHP2^{D61G} and GFP (lower) were expressed in cultured hippocampal neuron at DIV6 by using a bicistronic Sindbis virus vector. Scale bar, 20 μm . (C) Bar graph showing the average size (left) or the number (right) of GluN1-stained particles per 10 μm of dendrites. Unpaired *t*-test, ns, not significant. (D) Bar graph showing the average size (left) or the number (right) of GluA1-stained particles per 10 μm of dendrites. Unpaired *t*-test, * $p < 0.05$, ** $p < 0.01$. (E,F) Effect of PD98059 (PD: 10 μM) treatment on the average particle size (E) and the number (F) of surface GluN1. Two-way ANOVA followed by Bonferroni posttests. * $p < 0.05$, ** $p < 0.01$. Error bars represent standard error of the mean (SEM).

$n = 15$ neurons, 1451 μm of dendrites; Two-way ANOVA, interaction between genotype and treatment, $F_{1,81} = 9.506$, $p = 0.003$, Bonferroni posttest, ** $p < 0.01$; Fig. 1F, GluN1 particle number: SHP2^{D61G}, 13.93 ± 0.9 , $n = 25$ neurons, 2378 μm of dendrites; EGFP, 11.10 ± 0.6 , $n = 24$ neurons, 2207 μm of dendrites; SHP2^{D61G} + PD98059, 9.58 ± 0.7 , $n = 21$ neurons, 2012 μm of dendrites; EGFP + PD98059, 10.83 ± 1.1 , $n = 15$ neurons, 1451 μm of dendrites; Two-way ANOVA, interaction between genotype and treatment, $F_{1,81} = 5.756$, $p = 0.019$, Bonferroni posttest, * $p < 0.05$). It is worthy to note that 10 μM PD98059 was shown to be a sub-threshold dose which inhibits the excessively activated MAPK in cultured neuron without affecting neurotrophin-induced changes

in dendritic spines [17]. This result demonstrates that the mutant SHP2 increases the surface expression of NMDA receptors in a Ras-MAPK pathway dependent manner.

3.2. Mutant SHP2 overexpression at DIV 12 increases the size of AMPA receptor clusters

We infected cultured neurons with the Sindbis viruses encoding SHP2^{D61G} or EGFP at DIV 12 and analyzed the surface expression of glutamate receptors. In contrast to DIV 6, neurons infected at DIV 12 did not show significant differences in NMDA receptor expression in either the size or number of GluN1 clusters (Fig. 2A

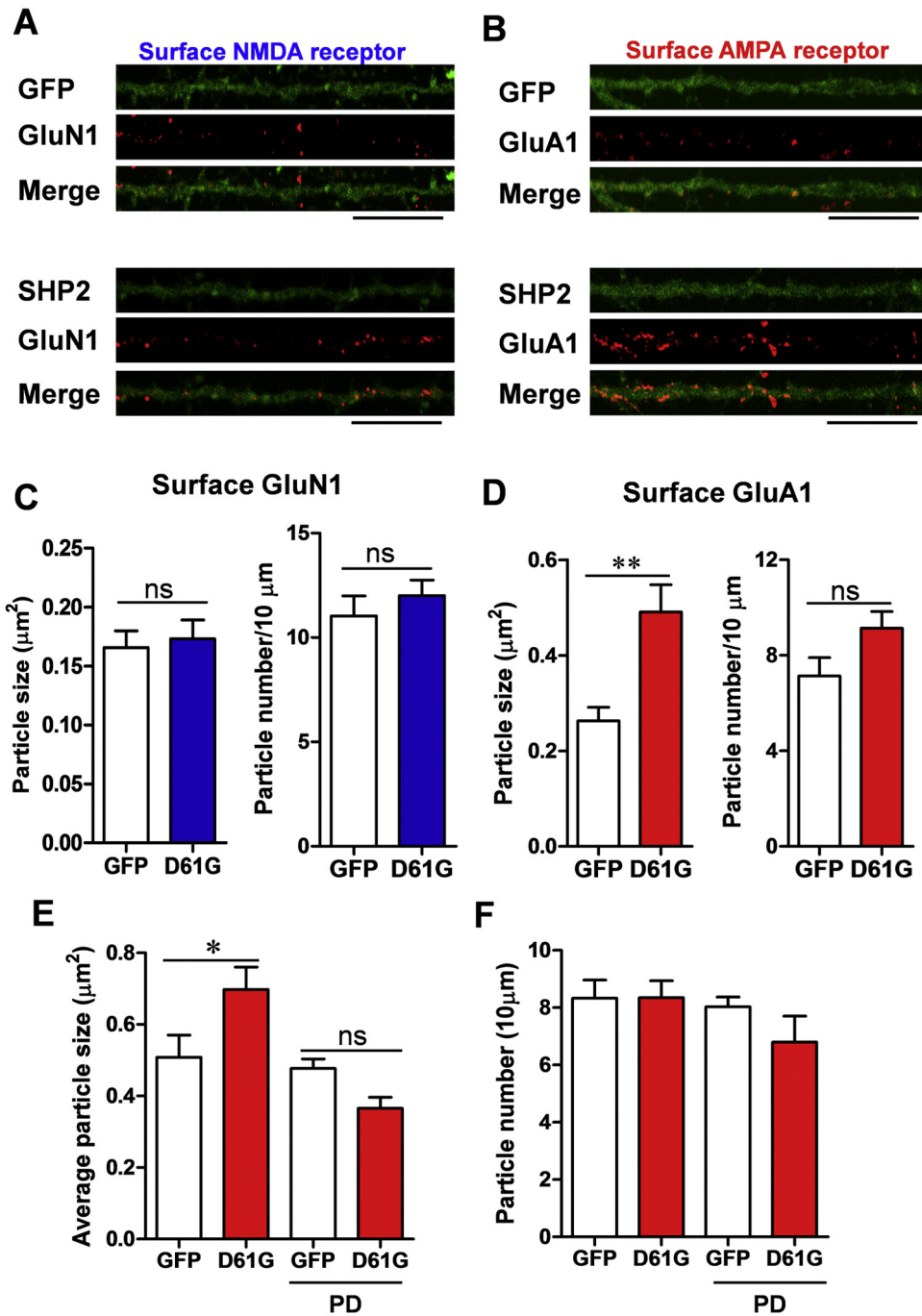


Fig. 2. SHP2^{D61G} overexpression at DIV 12 increases surface AMPA receptor expression. (A,B) Representative images of surface GluN1 (A) or GluA1 (B) receptor immunostaining. GFP alone (upper) or SHP2^{D61G} and GFP (lower) were expressed in cultured hippocampal neuron at DIV 12. Scale bar, 20 μm. (C) Bar graph showing the average size (left) or the number (right) of GluN1-stained particles per 10 μm of dendrites. Unpaired *t*-test, ** *p* < 0.01. (D) Bar graph showing the average size (left) or the number (right) of GluA1-stained particles per 10 μm of dendrites. Unpaired *t*-test, ns, not significant. (E,F) Effect of PD98059 (PD: 10 μM) treatment on the average particle size (E) and the number (F) of surface GluA1. Two-way ANOVA followed by Bonferroni posttests. * *p* < 0.05. Error bars represent SEM.

and C; GluN1 particle size: SHP2^{D61G}, 0.17 ± 0.0 μm², *n* = 16 neurons, 1418 μm of dendrites; EGFP, 0.17 ± 0.0 μm², *n* = 15 neurons, 1323 μm of dendrites; unpaired *t*-test, *p* = 0.727; GluN1 particle number per 10 μm: SHP2^{D61G}, 12.01 ± 0.7, *n* = 16 neurons, 1418 μm of dendrites; EGFP, 11.03 ± 1.0, *n* = 15 neurons, 1323 μm of dendrites; unpaired *t*-test, *p* = 0.422). However, we found that the size of GluA1 receptor clusters were significantly increased in SHP2^{D61G}-expressing neurons compared to EGFP controls (Fig. 2B and D; GluA1 particle size: SHP2^{D61G}, 0.49 ± 0.1 μm², *n* = 14 neurons, 1245 μm of dendrites; EGFP, 0.26 ± 0.0 μm², *n* = 14 neurons,

1220 μm of dendrites; unpaired *t*-test, ** *p* < 0.01). Interestingly, the numbers of GluA1 clusters were not statistically different between groups, although there was a trend toward an increase in the SHP2^{D61G} group (Fig. 2B and D; GluA1 particle number per 10 μm: SHP2^{D61G}, 9.14 ± 0.7, *n* = 14 neurons, 1245 μm of dendrites; EGFP, 7.14 ± 0.8, *n* = 14 neurons, 1220 μm of dendrites; unpaired *t*-test, *p* = 0.067). Importantly, PD98059 treatment completely normalized the size of GluA1 clusters in SHP2^{D61G}-expressing neurons, whereas it did not affect the number of GluA1 clusters (Fig. 2E, GluA1 particle size: SHP2^{D61G}, 0.70 ± 0.1 μm², *n* = 12 neurons, 1167 μm of den-

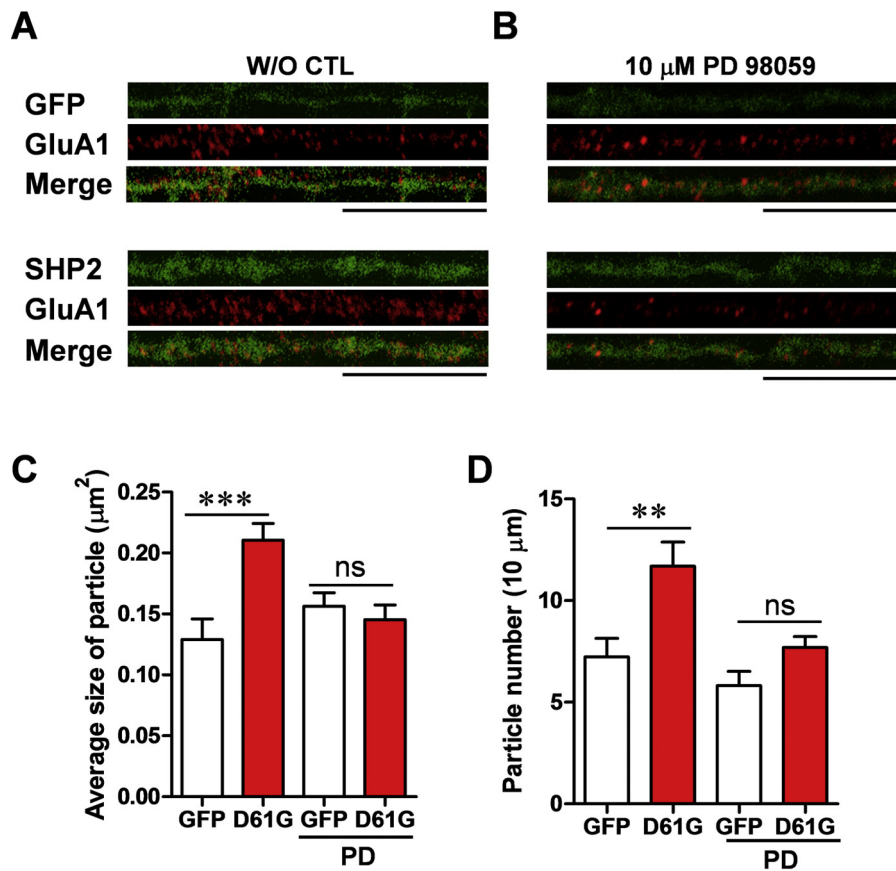


Fig. 3. SHP2^{D61G} overexpression at DIV 18 increases both the size and the number of the surface AMPA receptor clusters. (A,B) Representative images of surface GluA1 receptor staining in control (A) or PD98059-treated (B) cultured neurons. GFP alone (upper) or SHP2^{D61G} and GFP (lower) were expressed in cultured hippocampal neuron at DIV 18. Scale bar, 20 μm. (C,D) Effect of PD98059 (10 μM) treatment on the average particle size (C) and the number (D) of surface GluA1. Two-way ANOVA followed by Bonferroni posttests. ns, not significant, ** $p < 0.01$, *** $p < 0.001$. Error bars represent SEM.

drites; EGFP, $0.51 \pm 0.1 \mu\text{m}^2$, $n = 12$ neurons, 1239 μm of dendrites; SHP2^{D61G} + PD98059, $0.37 \pm 0.0 \mu\text{m}^2$, $n = 13$ neurons, 1336 μm of dendrites; EGFP + PD98059, $0.48 \pm 0.0 \mu\text{m}^2$, $n = 13$ neurons, 1229 μm of dendrites; two-way ANOVA, interaction between genotype and treatment, $F_{1,46} = 10.080$, $p = 0.0027$, Bonferroni posttest, * $p < 0.05$; Fig. 2F, GluA1 particle number: SHP2^{D61G}, 8.34 ± 0.6 , $n = 12$ neurons, 1167 μm of dendrites; EGFP, 8.37 ± 0.6 , $n = 12$ neurons, 1239 μm of dendrites; SHP2^{D61G} + PD98059, 6.80 ± 0.9 , $n = 13$ neurons, 1336 μm of dendrites; EGFP + PD98059, 8.02 ± 0.3 , $n = 13$ neurons, 1229 μm of dendrites; two-way ANOVA, interaction between genotype and treatment, $F_{1,46} = 0.911$, $p = 0.345$), suggesting that SHP2^{D61G}-mediated hyperactivation of the Ras-MAPK signaling pathway in moderately matured hippocampal neurons specifically increases the size of surface AMPA receptor clusters, without affecting NMDA receptor containing clusters. In addition, the total expression level of GluA1 was not changed by the expression of SHP2^{D61G} (Supplementary Fig. 1).

3.3. Mutant SHP2 overexpression at DIV 18 increases both the number and the size of AMPA receptor clusters

We examined the effect of SHP2^{D61G} overexpression on AMPA receptor expression in developing neurons at later stages. In fully mature neuron (DIV 18), both the size and the number GluA1-containing receptor clusters were significantly increased in SHP2^{D61G}-expressing neurons compared to EGFP-expressing control neurons (Fig. 3A, C and D). Consistently, SHP2^{D61G}-expressing neurons treated with PD98059 showed comparable size and number of GluA1 clusters to those in the EGFP control group

(Fig. 3A–C; GluA1 particle size: SHP2^{D61G}, $0.21 \pm 0.0 \mu\text{m}^2$, $n = 10$ neurons, 862 μm of dendrites; EGFP, $0.13 \pm 0.0 \mu\text{m}^2$, $n = 9$ neurons, 896 μm of dendrites; SHP2^{D61G} + PD98059, $0.15 \pm 0.0 \mu\text{m}^2$, $n = 15$ neurons, 1324 μm of dendrites; EGFP + PD98059, $0.16 \pm 0.0 \mu\text{m}^2$, $n = 11$ neurons, 1058 μm of dendrites; two-way ANOVA, interaction between genotype and treatment, $F_{1,41} = 11.62$, $p = 0.0015$, Bonferroni posttest, *** $p < 0.001$; Fig. 3D, GluA1 particle number: SHP2^{D61G}, 11.70 ± 1.2 , $n = 10$ neurons, 862 μm of dendrites; EGFP, 7.23 ± 0.9 , $n = 9$ neurons, 896 μm of dendrites; SHP2^{D61G} + PD98059, 7.70 ± 0.5 , $n = 15$ neurons, 1324 μm of dendrites; EGFP + PD98059, 5.82 ± 0.7 , $n = 11$ neurons, 1058 μm of dendrites; Two-way ANOVA, effect of treatment, $F_{1,41} = 10.940$, $p = 0.002$, Bonferroni posttest, ** $p < 0.01$), demonstrating that SHP2^{D61G} expression facilitates the surface expression of AMPA receptors in both the size and the number of GluA1-containing receptor clusters.

The results presented above demonstrate the differential effect of SHP2^{D61G} overexpression on glutamate receptor expression during development and show that decreasing MAPK signaling reverses abnormalities in the size and number of GluA1 and GluN1 clusters in SHP2^{D61G}-expressing neurons (Table 1).

4. Discussion

Mutations in the components of Ras-MAPK signaling pathway are closely associated with a group of genetic disorders, collectively called Rasopathies, that includes NS, neurofibromatosis, cardio-facio-cutaneous syndrome, LEOPARD syndrome and others [18]. Mutations in *PTPN11*, which encodes a non-receptor tyrosine phos-

Table 1
Differential effects of SHP2^{D61G} during maturation.

	Premature (DIV 6)		Mature (>DIV 12)		Adult (>DIV 21)	
	Size	Number	Size	Number	Size	Number
GluN1 particel	↑	↑	–	–	–	–
GluA1 particel	–	–	↑	↑	–	↑

↑, increase; –, no effect.

phatase SHP2, account for ~50% of NS cases. In the developing brain, SHP2 plays a pivotal role in determining the fate of neural progenitor cells [10,19]. SHP2 is also involved in the generation of oligodendrocyte progenitor cells [20,21]. We have reported that a NS-associated SHP2^{D61G} mutation impairs synaptic plasticity and subsequently disrupt hippocampal learning in adult mice, which can be reversed by inhibiting the activation of Ras-MAPK pathway [7]. We showed that AMPA receptor-mediated currents were significantly enhanced in adult NS mutants and that the surface expression of AMPA receptors was enhanced in SHP2^{D61G}-expressing cultured neurons at DIV 21 [7]. Consistently, a recent study also showed that SHP2 is critically involved in the surface expression of AMPA receptors using cultured neuron at DIV 18 [22]. In the present study, we also found that surface AMPA receptor expression is significantly increased in SHP2^{D61G}-expressing cultured neurons at DIV 18, which can be reversed by inhibiting MAPK signaling (Fig. 3). It is interesting that SHP2^{D61G} first increased the size of GluA1 (AMPA receptor) clusters at DIV 12 and later increased the number of GluA1 clusters at DIV 18 (Figs. 2 and 3). In the previous study, SHP2^{D61G} increased only the number of GluA1 clusters, but not the size in fully matured neurons at DIV 21 [7]. In this study, we choose DIV 18 to determine if changes in GluA1 expression were present prior to DIV 21. Altogether, the mutant SHP2 first increases the size of AMPA receptor clusters, presumably within functional spines, which may then affect multiple spines and subsequently increase the number of AMPA receptor clusters in fully matured neurons (Table 1).

Unexpectedly, we found that SHP2^{D61G} affects the surface expression of GluN1-containing NMDA receptor clusters only in premature neurons at DIV 6, but not in early maturing neurons at DIV 12 (Table 1). It is well known that NMDA receptor-mediated currents are larger than AMPA receptor currents in early developmental stages and that the number of synaptic AMPA receptors increase relative to the number of synaptic NMDARs during development [23]. Our data suggest that SHP2^{D61G} first increases NMDA receptor expression in a MAPK-dependent manner. Although NMDA receptor trafficking and expression can be regulated by the phosphorylation of receptor subunits [24], it is not yet clear how Ras-MAPK activation facilitates the surface expression of NMDA receptors. It has been reported that SHP2 is involved in BDNF-mediated upregulation of GluN2B NMDA receptors in the spinal dorsal horn after nerve ligation [25]. However, it is unknown whether BDNF/SHP2/GluN2B signaling also requires MAPK activation. It is well established that MAPK is critical for the regulation of activity-induced translation [26]. One possibility is that SHP2^{D61G}-mediated MAPK activation enhances the synaptic translation of NMDA receptor genes in premature neurons. Alternatively, SHP2^{D61G} may block the processes involved in either the endocytosis or the degradation of NMDA receptors. Further studies are required to clarify the underlying mechanism for the upregulation of NMDA receptor expression by SHP2^{D61G} in premature neurons. In addition, it is not clear whether the changes in GluN1 at DIV 6 may have any impact on the changes in GluA1 levels after DIV 12.

In summary, we found that NS-associated mutant SHP2^{D61G} differentially disrupt the composition of postsynaptic glutamate

receptor subtypes over development, which may interfere with the formation of normal functional connectivity in the brain and eventually contribute to the cognitive deficits in NS.

Conflicts of interest

The authors declare that they have no potential conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2017.03.036>.

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