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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.

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), $Cy^{TM}3$-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 μM 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: $SHP2^{D61G}$, $0.32 \pm 0.0\ \mu m^2$, $n = 15$ neurons, 1450 μm of dendrites; EGFP, $0.18 \pm 0.0\ \mu m^2$, $n = 20$ neurons, 1803 μm of dendrites; unpaired *t*-test, $^{**}\ p < 0.01$; GluN1 particle number per 10 μm: $SHP2^{D61G}$, 14.62 ± 1.0, $n = 15$ neurons, 1450 μm of dendrites; EGFP, 11.31 ± 0.8, $n = 20$ neurons, 1803 μm 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: $SHP2^{D61G}$, $0.12 \pm 0.1\ \mu m^2$, $n = 21$ neurons, 1894 μm of dendrites; EGFP, $0.11 \pm 0.0\ \mu m^2$, $n = 16$ neurons, 1394 μm of dendrites; unpaired *t*-test, $p = 0.768$; GluA1 particle number per 10 μm: $SHP2^{D61G}$, 8.66 ± 0.8, $n = 21$ neurons, 1894 μm of dendrites; EGFP, 10.97 ± 1.0, $n = 16$ neurons, 1394 μm 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 μM, 1 h) showed unchanged number and size of surface GluN1 particles compared to EGFP controls (Fig. 1E, GluN1 particle size: $SHP2^{D61G}$, $0.19 \pm 0.0\ \mu m^2$, $n = 25$ neurons, 2378 μm of dendrites; EGFP, $0.14 \pm 0.0\ \mu m^2$, $n = 24$ neurons, 2207 μm of dendrites; $SHP2^{D61G}$ + PD98059, $0.09 \pm 0.0\ \mu m^2$, $n = 21$ neurons, 2012 μm of dendrites; EGFP + PD98059, $0.12 \pm 0.0\ \mu m^2$,