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Research Paper

The Autism-Related Protein PX-RICS Mediates GABAergic Synaptic Plasticity in Hippocampal Neurons and Emotional Learning in Mice

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ABSTRACT

GABAergic dysfunction underlies many neurodevelopmental and psychiatric disorders. GABAergic synapses exhibit several forms of plasticity at both pre- and postsynaptic levels. NMDA receptor (NMDAR)–dependent inhibitory long-term potentiation (iLTP) at GABAergic postsynapses requires an increase in surface $GABA_A$Rs through promoted exocytosis; however, the regulatory mechanisms and the neuropathological significance remain unclear. Here we report that the autism-related protein PX-RICS is involved in $GABA_A$R transport driven during NMDAR–dependent GABAergic iLTP. Chemically induced iLTP elicited a rapid increase in surface $GABA_A$Rs in wild-type mouse hippocampal neurons, but not in *PX-RICS/RICS*–deficient neurons. This increase in surface $GABA_A$Rs required the PX-RICS/GABARAP/14–3-3 complex, as revealed by gene knockdown and rescue studies. iLTP induced CaMKII–dependent phosphorylation of PX-RICS to promote PX-RICS–14-3-3 assembly. Notably, *PX-RICS/RICS*–deficient mice showed impaired amygdala–dependent fear learning, which was ameliorated by potentiating GABAergic activity with clonazepam. Our results suggest that PX-RICS–mediated $GABA_A$R trafficking is a key target for GABAergic plasticity and its dysfunction leads to atypical emotional processing underlying autism.

1. Introduction

There is a growing consensus that autism arises from the atypical regulation of the excitation/inhibition balance within specific neural microcircuitry [1, 2]. In terms of neural inhibition, autism is closely related to dysfunctional inhibitory signaling mediated by the γ-aminobutyric acid (GABA) type A receptors ($GABA_A$Rs). Impaired presynaptic release of GABA and postsynaptic trafficking of $GABA_A$Rs lead to autistic-like social behavior in mouse models of autism [3–6]. There is a significant reduction in the number of $GABA_A$Rs and GABAergic activity in certain brain areas of autistic individuals [7–11]. Genetic association studies have revealed that several $GABA_A$R subunits are linked to an increased risk for autism [12–18]. $GABA_A$R–mediated signaling is thus essential for the proper regulation of the excitation/inhibition balance associated with socio-emotional cognition.

Similar to glutamatergic synapses, GABAergic synapses also exhibit activity–dependent plastic changes in their inhibitory transmission efficacy. Different forms of GABAergic synaptic plasticity based on diverse pre- and post-synaptic mechanisms have been characterized in different brain regions [19–22]. A major postsynaptic form of GABAergic synaptic plasticity is *N*-methyl-D-aspartate receptor (NMDAR)–dependent inhibitory long-term potentiation (iLTP) [21, 23–25], which is elicited by moderate stimulation of NMDARs that induces limited Ca^{2+} influx and preferential activation of Ca^{2+}/calmodulin–dependent protein kinase II (CaMKII). Activated CaMKII translocates to inhibitory synapses [26], where it triggers *de novo* $GABA_A$R transport to the neuronal surface, resulting in a persistent increase in the number of surface-expressed $GABA_A$Rs and inhibitory synaptic transmission. This increase in surface $GABA_A$Rs involves $GABA_A$R-associated protein (GABARAP), *N*-ethylmaleimide-sensitive factor (NSF) and glutamate receptor interacting protein (GRIP) [27]; however, the precise molecular mechanisms remain obscure. In particular, the relevant trafficking-related proteins that are phosphorylated by CaMKII and facilitate surface expression of $GABA_A$Rs are currently unidentified.

We have previously identified and characterized two splicing isoforms of GTPase-activating proteins specific for Cdc42 predominantly expressed in neurons of the cerebral cortex, amygdala and hippocampus: RICS (ARHGAP32 isoform 2) and PX-RICS (ARHGAP32 isoform 1) [28, 29]. RICS regulates NMDAR–mediated signaling at the postsynaptic density and axonal elongation at the growth cone [29, 30]. In contrast, PX-RICS forms an adaptor complex with GABARAP and 14-3-3ζ/θ to facilitate steady-state trafficking of the N-cadherin/β-catenin complex and $GABA_A$Rs [3, 28, 31, 32]. *PX-RICS* is also responsible for autistic-like features observed in more than half of the patients with Jacobsen syndrome (JBS) [3]. Mice lacking *PX-RICS/RICS* show marked decreases in surface-expressed $GABA_A$Rs and $GABA_A$R–mediated inhibitory synaptic transmission, resulting in various autistic-like behaviors

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and autism-related comorbidities [3]. Rare single-nucleotide variations in *PX-RICS* are also linked to non-syndromic autism, schizophrenia and alexithymia [33–35]. These findings strongly suggest that dysfunction of PX-RICS–mediated $GABA_AR$ trafficking has severe effects on socio-emotional processing of the brain.

Our previous study described above showed that PX-RICS and other components of the $GABA_AR$ trafficking complex are required for constitutive transport of the receptor. In this study, we have focused on the role of PX-RICS in the activity–induced promotion of $GABA_AR$ trafficking during iLTP. Here we show that PX-RICS–mediated $GABA_AR$ trafficking is also involved in NMDAR activity–dependent trafficking of $GABA_ARs$ and that PX-RICS is a key target of CaMKII for regulating GABAergic synaptic plasticity. Furthermore, we show that PX-RICS dysfunction in mice leads to impaired amygdala–dependent emotional learning, which manifests as autistic-like social behavior [3].

2. Materials and Methods

2.1. Mice

All animal experiments were reviewed and approved by the University of Tokyo Institutional Animal Care and Use Committee and were conducted according to the University of Tokyo Guidelines for Care and Use of Laboratory Animals. Mice were housed in clean plastic cages (CLEA Japan) lined with paper bedding (Japan SLC) at a constant temperature of 23 °C with a 12-h light/dark cycle (lights off at 21:00), with food and water available *ad libitum*. *PX-RICS*/*RICS*–deficient mice were generated as described [30]. Mutant mice were backcrossed into the C57BL/6 N (CLEA Japan) background until the F10 generation. The mice used in behavioral studies were generated by breeding heterozygous mutant males and females in the C57BL/6 N background. Adult and young-adult male mice (20 and 8–10 weeks old, respectively) from a naïve cohort were used in the fear conditioning and pain sensitivity tests. The embryos for primary neuronal cultures were generated by crossing wild-type C57BL/6 N males and females, or homozygous mutant males and females in the C57BL/6 N background. Embryonic day (E) 16–18 embryos were used for primary culture of hippocampal neurons.

PX-RICS/*RICS*–deficient mice carry the disrupted *Arhgap32* gene encoding PX-RICS and RICS, two splicing isoforms with the distinct cellular functions [3, 28, 29, 31, 32]. Autistic-like behaviors of the mutant mice are reversed by a $GABA_AR$ agonist clonazepam, suggesting that these phenotypes are caused by PX-RICS dysfunction, not by RICS deficiency [3]. For this reason, the KO mice were termed *PX-RICS* KO in our previous studies. In this study, however, the KO mice were accurately termed *PX-RICS*/*RICS* KO.

2.2. Cell Culture and Transfection

Hippocampal neurons were isolated from E16–18 mouse embryos and plated on 24-well tissue culture plates precoated with 1 mg/ml poly-L-lysine (Sigma-Aldrich) as described [29]. Cells were cultured in Neurobasal medium (Thermo Fisher Scientific) supplemented with B-27 supplement (Thermo Fisher Scientific) and 0.5 mM L-glutamine (Thermo Fisher Scientific). For the first 3 days in culture, 10 μM cytosine β-D-arabinofuranoside (Ara-C; Sigma-Aldrich) was included in the culture medium. Half of the medium was changed every 3 days. Transfection of primary cultured neurons was performed at 14 days *in vitro* (DIV) using FuGENE 6 (Roche) for plasmid constructs and 10 DIV using Lipofectamine RNAiMAX (Thermo Fisher Scientific) for siRNAs. Three hours after transfection, the medium was replaced with conditioned medium.

2.3. Chem-iLTP Induction

Induction of chem-iLTP was performed as described [27]. Briefly, mouse hippocampal neurons in culture were treated with 20 μM NMDA (Sigma-Aldrich) plus 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma-Aldrich) for 3 min at 37 °C followed by recovery incubation in conditioned medium for 12 min at 37 °C. The cell-permeable, water-soluble CaMKII inhibitor KN93 [36] (1 μM; Merck Millipore) was applied for 30 min prior to NMDA treatment and also was added to the medium for NMDA stimulation and for recovery incubation. Neurons were then subjected to immunoblotting, immunoprecipitation or surface labeling.

2.4. Antibodies

A rabbit polyclonal antibody (pAb) against PX-RICS was generated as described [28]. A rabbit pAb specific for the $GABA_AR$ β3 subunit phosphorylated at Ser^{383} (phospho-S^{383}) was a kind gift from Dr. Stephen J. Moss (Department of Neuroscience, Tufts University School of Medicine) [37, 38]. Commercially available antibodies used for immunoblotting were as follows: rabbit monoclonal antibody (mAb) against CaMKII (1:1000; Cell Signaling Technology, #4436, lot: 3), rabbit mAb against phospho-CaMKII (Thr^{286}) (1:1000; Cell Signaling Technology, #12716, lot: 3), rabbit pAb against GluA1 (1:600; Abcam, ab31232, lot: GR79640-1), rabbit pAb against phospho-GluA1 (Ser^{845}) [1:500; Upstate Biotechnology, #06-773 (currently AB5849, Merck Millipore), lot: 23869A], mouse mAb against α-tubulin (1:500; Merck Millipore, CP06, lot: D00160163), rabbit pAb against 14-3-3ζ (1:100; Santa Cruz Biotechnology, sc-1019, lot: C1008), mouse mAb against 14-3-3θ (1:5000; Sigma-Aldrich, T5942, lot: 107 K1655), and mouse mAb against $GABA_AR$ β3 subunit (1:1000; Synaptic Systems, #224411, lot: 224411/1). Commercially available antibodies used for immunofluorescent staining were as follows: rabbit pAb against the $GABA_AR$ γ2 subunit (1:500; Synaptic Systems, #224003, lot: 224003/8) and mouse mAb against vesicular GABA transporter (VGAT) (1:1000; Synaptic Systems, #131011, lot: 131011/26).

2.5. Immunoprecipitation and Immunoblotting

Mouse hippocampal neurons (14 DIV) were lysed in lysis buffer T [10 mM Tris-HCl (pH 6.8), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 with protease/phosphatase inhibitor cocktail (Sigma-Aldrich)]. The lysates were precleared with protein A–Sepharose (GE Healthcare) for 1 h at 4 °C. Precleared lysates (500 μg of protein) were incubated with 5 μg of the indicated antibody for 1 h at 4 °C, and then the immunocomplexes were adsorbed to protein A–Sepharose for 1 h at 4 °C. After being washed extensively with lysis buffer T, the immunoprecipitates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck Millipore). The blots were probed with primary antibodies as indicated and visualized with alkaline phosphatase–conjugated secondary antibodies (Promega). Band intensities were quantified using ImageJ software.

2.6. Phos-tag SDS-PAGE

Phos-tag SDS-PAGE [6% acrylamide containing 50 μM Phos-tag acrylamide (Wako)] and immunoblotting were performed according to the manufacturer's instructions.

2.7. Labeling of Surface and Internal γ2 Subunits

Surface labeling was performed as described [3]. The rabbit pAb against γ2 (Synaptic Systems) used for surface labeling recognizes the N-terminal extracellular region of the subunit. Briefly, primary and secondary antibodies used for surface labeling before fixation were diluted in conditioned medium from 14 DIV hippocampal neurons. Mouse hippocampal neurons (14 DIV) were surface-labeled with anti–γ2 (1:500) for 30 min at room temperature and rinsed in phosphate-buffered saline (PBS). The neurons were then incubated with Alexa Fluor 488–conjugated anti–rabbit IgG (1:500; Thermo Fisher Scientific) for

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