Regulatory mechanisms of brain and neuronal functions revealed through comprehensive identification of mRNAs localized to dendrites by RNG105 (Caprin1)

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Introduction

Spatiotemporal translational regulation plays important roles in increasing local concentrations of specific proteins to exert their functions at specific timing and location in cells. In neurons, mRNA transport and local translation in dendrites play crucial roles in long-lasting synaptic plasticity and higher-order brain functions (Kang and Schuman, 1996; Costa-Mattioli et al., 2009). RNA granules are key macromolecular complexes for dendritic mRNA transport and synaptic stimulation-dependent translational control (Knowles et al., 1996; Kiebler and Bassell, 2006). Translational regulators and RNA-binding proteins including the RNA granule components are emerging as factors associated with neurodevelopmental disorder, intellectual disability and mental disorder, e.g., the mammalian target of the rapamycin complex 1 (mTORC1) signaling pathway in autism spectrum disorder (ASD), the fragile X mental retardation protein (FMRP) in fragile X syndrome, and disrupted-in-schizophrenia 1 (DISC1) in schizophrenia (Buffington et al., 2014; Lenzken et al., 2014; Santos et al., 2014; Tsuboi et al., 2015).

In this study, I focused on one of the RNA-binding proteins in RNA granules,

RNA granule protein 105 (RNG105, also known as Caprin1). RNG105 is a major RNA-binding protein in the RNA granules and most highly expressed in the brain (Shiina et al., 2005; Shiina and Tokunaga, 2010). RNG105 is responsible for the dendritic transport of its binding mRNAs in primary cultured neurons *in vitro* (Shiina et al., 2010). In addition, RNG105 knockout impairs synapse formation on dendrites, development of dendrites, and neuronal network formation *in vitro* (Shiina et al., 2010), suggesting that RNG105 regulates the synaptic and neuronal network functions. However, it was unclear whether RNG105 is associated with neurodevelopmental and mental disorders.

A recent study aiming to detect genetic variants in ASD by whole-genome sequencing reported that a heterozygous *de novo* nonsense mutation in the *Rng105* (*Caprin1*) gene was found in a patient with Asperger's syndrome (Jiang et al., 2013). The patient's intelligence quotient (IQ) was above average, but adaptive behavior was below average and sociability was delayed (Jiang et al., 2013). The results suggested that *Rng105* (*Caprin1*) is a candidate risk gene for ASD, but it remains unclear as to whether there is a causal relation between RNG105 deficiency and ASD. In the present study, to investigate the influence of RNG105 deficiency on mouse behavior, I subjected RNG105 heterozygous ($Rng105^{+/-}$) mice to a comprehensive behavioral test battery.

Another question is whether RNG105 is responsible for the dendritic localization of mRNA *in vivo*, and if so, what kind of mRNA is localized to dendrites by RNG105 and how RNG105 impacts on higher-order brain function through regulating the dendritic mRNA localization. In this study, I comprehensively analyzed the somato-dendritic mRNA distribution in the hippocampus of RNG105 conditional knockout (cKO) mice that had shown field excitatory postsynaptic potential (fEPSP) amplitude reduction and long-term memory impairment. The mRNA distribution analysis identified a new panel of dendritic mRNAs, which provided a new insight into the mechanism of synapse formation and plasticity regulated through dendritic mRNA

localization.

Material and Methods

Animals and design of behavioral test battery

Rng105$^{+/-}$ mice were generated previously (Shiina et al., 2010), and backcrossed for more than 10 generations on the C57BL/6 background. *Rng105*$^{+/+}$ mice and *Rng105*$^{+/-}$ mice were subjected to a comprehensive behavioral test battery. All behavioral tests were carried out with male mice. Mice were group-housed (4 mice per cage, two pairs of wild-type and *Rng105*$^{+/-}$ mice) in a room with a 12-hour light/dark cycle. The order in which mice were subjected to tests was counterbalanced.

Tissue dissection and isolation of SP and SR from hippocampus

Mouse brains (postnatal 12 weeks old) were removed and coronal sections (500 μm thick) were sliced using a vibratome (VT1200S, LEICA, Wetzlar, Germany) in ice-cold sterile phosphate buffered saline (PBS). Stratum pyramidale (SP) and stratum radiatum (SR) in the hippocampal CA1 region were microdissected manually using glass capillaries with the use of a stereomicroscope. The borders between the stratum oriens (SO) and SP, and between the SP and SR were cut to isolate the SP, and the border between SP and SR, and between SR and stratum lacnosum-moleculare (SLM) were cut to isolate the SR. To obtain sufficient amount of RNA for RNA-seq, left and right hippocampi from 3 mice were dissected and collected into one sample tube. Triplicate samples were prepared for RNA-seq analysis.

RNA extraction and preparation of cDNA libraries for RNA-seq

Total RNA was extracted from the tissues (SP and SR) using ISOGEN (Nippon gene, Tokyo, Japan) in accordance with the manufacturer's protocols with minor

modifications. DNA was removed from the sample by DNase treatment using DNase (RT grade) (Nippon Gene). Total RNA (200 ng per sample) was used as the starting material to prepare cDNA using TruSeq RNA sample Preparation Kit v2 (Illumina, San Diego, CA, USA) at a half scale compared with the manufacturer's protocols.

RNA-seq and read alignment

In each experiment, an equal amount of cDNA from SP and SR was analyzed, and three independent experiments were conducted. Twelve cDNA libraries (four kinds of samples × three biological replicates) were sequenced with 101 bp paired-end sequencing using a HiSeq1500 (Illumina, San Diego, CA, USA). The resulting reads were mapped to the mouse genome (*Mus musculus* Ensemble NCBIM37) using TopHat (v 2.0.11). The mapped reads were assembled using Cufflinks (v 2.2.1), and differential gene enrichment analysis was conducted using Cuffdiff.

Gene ontology analysis of RNA-seq data

Gene ontology enrichment analysis was performed using DAVID functional annotation tools. Significance of overrepresentation of GO terms was assessed using the Benjamini-Hochberg false discovery rate (FDR) criterion at $p < 0.05$.

Results

Rng105$^{+/-}$ mice exhibited reduced sociality in a home cage in 24 h home cage social interaction test and reduced preference for social novelty in three-chambered social approach test. These results suggested that the mice reduced sociality and response to novelty. The reduced response to novelty was consistent with that *Rng105*$^{+/-}$ mice showed reduced preference for novel objects in novel object recognition test and novel place patters in place recognition test. Regarding learning and memory, although

Rng105$^{+/-}$ mice exhibited normal memory acquisition in Barnes maze test, T-maze test and contextual fear conditioning test, they tended to have relative difficulty in reversal learning in spatial reference tasks. These findings suggested that RNG105 heterozygous knockout leads to a reduction in sociality, response to novelty and flexibility in learning, which are implicated in ASD-like behaviors.

In neuronal cell level analysis, RNG105-deficient neurons showed a reduction in AMPA glutamate receptor (AMPAR) cell surface expression in dendrites, which has been reported in other ASD-like mutant mice and thought to be related with the neuropathology of ASD (Nakamoto et al., 2007; Wang et al., 2011; O'connor et al., 2014). The behavioral test battery, together with the analysis of the AMPAR surface expression, suggested that an RNG105 deficiency led to an ASD-like phenotype.

Next, I investigated the impact of RNG105 deficiency on mRNA localization in the mouse hippocampus. Genome-wide profiling of mRNA distribution in the hippocampus was conducted by dissecting somatic and dendritic layers followed by next-generation RNA-seq analysis in RNG105 cKO mice and control mice. I identified 1,122 dendritically-enriched mRNAs and 2,106 somatically-enriched mRNAs in control mice. The dendritically-enriched mRNAs included already known dendritic mRNAs such as *Camk2a*, *Eef1a1*, *Dlg4*, *Iptr1*, *Arc*, *Shank2*, *Homer2*, and *Limk1*, suggesting that the strategy was appropriate to detect somato-dendritic mRNA distribution pattern.

Although control mice showed asymmetric somato-dendritic localization of mRNA in the hippocampus, this asymmetric distribution pattern was impaired by RNG105 cKO. Dendritically-enriched mRNAs in control mice were mainly categorized by gene ontology (GO) analysis in "regulation of Arf protein signal transduction" and "structural constituent of ribosomes", and mRNAs categorized in these GO categories were reduced in the dendritic layer of RNG105 cKO mice. Particularly, RNG105 cKO reduced the dendritic localization of mRNAs encoding regulators of AMPAR cell

surface expression. Consistent with this, RNG105 knockout in primary cultured neurons from mouse brains reduced surface AMPARs and attenuated homeostatic AMPAR scaling in dendrites. These results suggested that RNG105 is required for synaptic potentiation such as AMPAR surface expression through regulating dendritic mRNA localization, which is considered to be an underlying mechanism of ASD-like behavior and the loss of long-term memory in RNG105-deficient mice.

Discussion

It is well known that ASD is a complex neurodevelopmental disorder (Silverman et al., 2010; Ey et al., 2011). However, ASD is mainly characterized by three phenotypes: abnormal social interactions, communication deficits and repetitive behaviors (Silverman et al., 2010; Crawley 2007). The first feature of ASD, i.e., abnormal social interactions, was detected in *Rng105*$^{+/-}$ mice. *Rng105*$^{+/-}$ mice showed a lack of social novelty preference and reduced social interaction. However, it is unclear whether the second feature of ASD, i.e., communication deficits, is shown in *Rng105*$^{+/-}$ mice. I could not measure the communication ability of *Rng105*$^{+/-}$ mice from the sociability tests such as the social interaction test in a home cage, three-chambered social approach test or the social interaction test in a novel environment. It is necessary to measure ultrasonic vocalization and olfaction in order to investigate communication in *Rng105*$^{+/-}$ mice. The third feature of ASD, i.e., repetitive behaviors, includes stereotyped behaviors, restricted interests and insistence on sameness (Silverman et al., 2010; Crawley 2007). The stereotypic behaviors of *Rng105*$^{+/-}$ mice in the open-field test were not significantly different from wild-type mice, but *Rng105*$^{+/-}$ mice had difficulties in the reversal learning tasks. Reversal learning tasks measure flexible response to changes and are considered to be assays for insistence on sameness (Silverman et al., 2010; Crawley 2007). Although *Rng105*$^{+/-}$ mice did not stick to the initial correct hole

or arm after the reversal trials in the Barnes maze or T-maze (left-right discrimination task) tests, they had specific impairments in reversal learning, but not in the initial acquisition, suggesting that the phenotypes of *Rng105*$^{+/-}$ mice were related to ASD-like behaviors in terms of upset to change.

The analysis of somato-dendritic mRNA distribution with RNA-seq in this study was the first application of the technique to mutant animals. The RNA-seq revealed that the loss of RNG105 reduced the asymmetric somato-dendritic localization of mRNAs *in vivo*. Furthermore, the RNA-seq identified multiple mRNAs whose dendritic localization was reduced by RNG105 deficiency, which included mRNAs encoding regulators of the cell surface expression and postsynaptic retention of AMPARs, such as regulators of Arf signal transduction. In addition, the identified mRNAs included those encoding Na^+/K^+ ATPase subunit isoforms and K^+ channel subunits, which was consistent with the previous *in vitro* study (Shiina et al., 2010). Proteins encoded by these mRNAs are involved in the control of membrane potential, and thereby may be associated with the reduced fEPSP amplitude and epileptic-like EPSP in RNG105 cKO mice. Furthermore, the identified mRNAs included those encoding regulators of Ras, Rho, and the PI3 kinase and Rac pathway proteins, involved in actin reorganization and spine formation (Nishiyama and Yasuda, 2015; Sala and Segal, 2014), which may be associated with the impaired structural plasticity of spines in RNG105 cKO mice. mRNAs for ribosomal subunit proteins were the major dendritic mRNAs and also reported in previous studies (Cajigas et al., 2012; Ainsley et al., 2014). However, whether locally translated ribosomal proteins are involved in ribosome biogenesis or in other biological processes, which could be associated with RNG105-defcient phenotypes, remains elusive. Thus, RNG105-dependent dendritic mRNAs included various mRNAs whose encoded proteins are involved in AMPAR localization, membrane potential control, and actin reorganization. Even if the reduction of each mRNA in dendrites could have a small influence on synaptic functions, integration of the reduction of these mRNAs could have a large impact on it.

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