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Research Paper

Reggie-1 and reggie-2 (flotillins) participate in Rab11a-dependent cargo trafficking, spine synapse formation and LTP-related AMPA receptor (GluA1) surface exposure in mouse hippocampal neurons

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ABSTRACT

Reggie-1 and -2 (flotillins) reside at recycling vesicles and promote jointly with Rab11a the targeted delivery of cargo. Recycling is essential for synapse formation suggesting that reggies and Rab11a may regulate the development of spine synapses. Recycling vesicles provide cargo for dendritic growth and recycle surface glutamate receptors (AMPAR, GluA) for long-term potentiation (LTP) induced surface exposure. Here, we show reduced number of spine synapses and impairment of an in vitro correlate of LTP in hippocampal neurons from reggie-1 k.o. (Flot2 −/−) mice maturating in culture. These defects apparently result from reduced trafficking of PSD-95 revealed by live imaging of 10 div reggie-1 k.o. (Flot2 −/−) neurons and likely impairs co-transport of cargo destined for spines: N-cadherin and the glutamate receptors GluA1 and GluN1. Impaired cargo trafficking and fewer synapses also emerged in reggie-1 siRNA, reggie-2 siRNA, and reggie-1 and -2 siRNA-treated neurons and was in siRNA and k.o. neurons rescued by reggie-1-EGFP and CA-Rab11a-EGFP. While correlative expressional changes of specific synapse proteins were observed in reggie-1 k.o. (Flot2 −/−) brains in vivo, this did not occur in neurons maturating in vitro. Our work suggests that reggie-1 and reggie-2 function at Rab11a recycling containers in the transport of PSD-95, N-cadherin, GluA1 and GluN1, and promote (together with significant signaling molecules) spine-directed trafficking, spine synapse formation and the in vitro correlate of LTP.

1. Introduction

The formation of the axon and elaboration of dendritic arborisation with spines and synapses are regulated by transport of membrane/membrane proteins to diverse neuronal functional domains. All stages of process development, maturation and adaptive changes of plasticity require membrane and protein recycling and make use of fundamental eukaryotic trafficking mechanisms (Park et al., 2004, 2006; Schulte et al., 2010). Indeed, the recycling endosome is involved in the targeted delivery of membrane and proteins to specific sites in the cells (Park et al., 2004, 2006; Solis et al., 2013; Takahashi et al., 2012). In maturated neurons, the recycling endosome contributes to spine growth at glutamatergic synapses and preserves the complex composition of the postsynaptic density (PSD) which harbors cell adhesion proteins, transmitter receptors and molecular scaffolds in register with the presynaptic transmitter-releasing axon protrusions (Kennedy and Ehlers, 2011; Arikkath and Reichardt, 2008). Recycling vesicles not only provide specific compounds for axon and dendritic growth but can serve as reservoir for growth of the PSD and spines, and supply AMPA type glutamate receptors (GluA) for constitutive GluA recycling and long-term potentiation (LTP) induced GluA1 surface exposure (Brown et al., 2007; Park et al., 2004, 2006; Kennedy and Ehlers, 2011). Blocking the transport of the Rab11 recycling endosome not only decreases GluA but also disrupts NMDA receptor (GluN)-dependent delivery of GluA1 to the surface (Park et al., 2004), which impairs LTP.

The lipid raft proteins reggie-1/flotillin-2 and reggie-2/flotillin-1 – which reside at synapses (Solis et al., 2010; Suzuki et al., 2011) – were found to bind Rab11a and to participate in recycling and the targeted delivery of membrane/membrane proteins to strategically important

Abbreviations: CA, constitutive active; ECS, extracellular solution; EGFP, enhanced green fluorescent protein; EHD, Eps15 homology domain-containing proteins; Flot2, flotillin-2; k.o., knock out; LTP, long term potentiation; MAPK, mitogen-activated kinase; div, days in culture; GluN1, NMDA receptor subunit 1 (NMDA receptor, *N*-Methyl-D-Aspartate receptor); GluA1, glutamate receptor subunit 1 (AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor); RFP, red fluorescent protein; FM4-64FX, fixable analog of FM® 4–64 membrane stain; siRNA, small interfering RNA; pMAPK, phosphorylated MAPK; JNK, Jun kinase; pJUNK, phosphorylated JNK; mAb, monoclonal antibody; pAb, polyclonal antibody; PFA, paraformaldehyde; PBS, phosphate buffered saline; PSD, postsynaptic density; PSD-95, postsynaptic density protein 95; pPSD-95, phosphorylated PSD-95; s.e.m., standard error of the mean; SNX4, sorting nexin4; wt, wild type.

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sites in diverse cells (Stuermer, 2010; Solis et al., 2013). They are essential for growth cone elongation and regeneration in retinal ganglion cells and mouse hippocampal neurons (Munderloh et al., 2009; Koch et al., 2013). They promote the targeted delivery of N-cadherin to the growth cone (Bodrikov et al., 2011) and regulate the targeted recycling of important cell surface proteins to distinct sites of the cell, E-cadherin to adherens junctions (Solis et al., 2013), α5- and β1-integrin to focal adhesions (Hülsbusch et al., 2015), the T cell receptor to the T cell cap (Stuermer et al., 2004, Stuermer, 2010). The targeted delivery seems to be promoted by the interaction of reggie-1 with Rab11a at the recycling compartment (Solis et al., 2013).

That reggies are involved in important steps of synapse formation and plasticity was suggested by reports showing by mass spectrometry analyses that flotillin-1/reggie-2 is upregulated during learning-related events in Aplysia and during spatial memory formation in the mouse hippocampus (Monje et al., 2013). Flotillin-1/reggie-2 is downregulated in the cortical barrel fields after sensory deprivation (Butko et al., 2013). Upregulation of flotillin-1/reggie-2 has, in addition, been implicated in the formation of glutamatergic synapses of mouse hippocampal neurons in vitro (Swanwick et al., 2010) and increase of the frequency of miniature excitatory postsynaptic currents.

Here, we investigated whether reggies participate in Rab11a-dependent trafficking of vesicular carriers in young and maturated mouse hippocampal neurons in culture and whether such trafficking defects would impair spine synapse formation by using neurons from reggie-1 knock out (k.o.; Flot2 −/−) mice (Berger et al., 2013) and neurons after siRNA-mediated reggie downregulation. Indeed, we find that reduced expression of reggie impairs trafficking of PSD-95 (and reggie)-decorated vesicular carriers in hippocampal neurons and reduces pre- and postsynaptic contacts (synapses) in spines. Accordingly, immunostaining intensity for the surface-exposed AMPAR subunit GluA1 was significantly reduced in dendrites particularly after glycine induction of an in vitro correlate of LTP. The reduced spine density in reggie-deficient neurons was rescued by overexpression of constitutive-active (CA) Rab11a consistent with our earlier evidence showing that reggie and Rab11a act in the same pathway for the targeted delivery/recycling (Solis et al., 2013; Stuermer, 2010), which in the present neurons affects PSD-95 and associated cargo destined for the spine synapse.

2. Material and methods

2.1. Animals

Reggie-1 knock-out (k.o.; Flot2 −/−) C57BL/6J mice were generously provided by Tak W. Mak (The Campbell Family Institute for Breast Cancer Research, University Toronto, Canada; Berger et al., 2013). K.o. and wild type (wt) C57BL/6J mice were raised in the animal facility, University of Konstanz. Male and female mice, 1–2 d and 2 months old, were used.

2.2. Antibodies

Monoclonal (mAbs) and polyclonal (pAbs) antibodies were: mAb against reggie-1 (1:500, ESA-29; Cat#610383, BD Biosciences Transduction Laboratories), pAb against reggie-1 (1:1000, H-90, Cat#sc-25507, Santa Cruz), mAb against reggie-2 (1:500, F-3, Cat#sc-74567, Santa Cruz), mAbs and pAbs against fyn (1:1000, E-3, Cat#sc-365913 and H-80, Cat#sc-28791, Santa Cruz), active fyn pAb (1:1000, Tyr-527-dephosphorylated, Cat#2107S, Cell Signaling Technology), pAbs N-cadherin (1:1000, H-63, Cat#sc-7939, Santa Cruz), pAbs against MAPK/ERK1/2 and pMAPK/pERK1/2 (1:1000, H-72, Cat#sc-292838 and p-ERK, 1:1000, Thr 202/Tyr 204, Cat#sc-16982, Santa Cruz), pAb against GluN1 (NMDAR1, 1:1000, D65B7, Cat# 5704), pAb against SAPK/JNK (Cat#9252), pAb against T183/Y185-SAPK/JNK (81E11, Cat# 4668), pAb against Rab11a (1:500, Cat#2413) and pAb against PSD-95 (1:1000, Cat. N#2507, Cell Signaling Technology), pAb against GluA1 (AMPAR1, 1:500, Cat#PC246-100UG, Merck Millipore), pAb against Rab5a (1:500, Cat#PAB9011, Abnova), mAb against synaptophysin (1:500, SY38, Cat#ab8049, Abcam) and pAb against S295-PSD-95 (1:500, EP2615Y, Cat#ab76108, Abcam). Secondary antibodies rabbit and mouse Ig coupled to HRP, Cy3, or Cy5 were from Dianova. Alexa 488 phalloidin was from Invitrogen.

2.3. Culture of hippocampal neurons

Hippocampal neurons from 1 to 2 d old wt C57BL/6J and reggie-1 k.o. (Flot2 −/−) mice (Berger et al., 2013) were exposed to papain (Sigma-Aldrich), 30 min, 37 °C, centrifuged (80 × *g*, 5 min, 37 °C), resuspended and seeded on poly-D-lysine (PDL) coated coverslips (12-well plates, Neurobasal A with B-27 serum-free supplement, Life Technologies, and 5 μg/ml FGF). After medium change, FGF was increased (10 μg/ml). Cells grew at 37 °C and 5% CO_2 (Bodrikov et al., 2011).

After fixation, neurons (48 h or 96 h) on glass coverslips were stained with Alexa 488 phalloidin (Invitrogen) to measure neurite length (NIH ImageJ software).

2.4. DNA constructs and transfection

The reggie-1 and reggie-2 siRNAs and the GL2 control siRNA were described previously (Munderloh et al., 2009; Solis et al., 2007). Alexa Fluor 546–labeled siRNA duplexes against reggie-1 (R1.0), reggie-2 (R2.0) and firefly luciferase (GL2, served as nonspecific control) were obtained from Dharmacon. The target sequences were for reggie-1: 5′-GTTCATGGCAGACACCAAG-3′ (R1.0), and for reggie-2: 5′-CACACTGA CCCTCAATGTC-3′ (R2.0) and used in wt neurons at concentrations of 9 pmol/ml (reggie-1 and reggie-2, respectively, and GL2) or each at 5 pmol/ml (when reggie-1 and reggie-2 siRNAs were combined, with the GL2 control at 10 pmol/ml). Cells were co-transfected with a plasmid encoding PSD-95-EGFP, reggie-1-EGFP, reggie-1-RFP, reggie-2-EGFP, CA-Rab11a-EGFP, CA-Rab11a-RFP or EGFP to identify transfected cells (Solis et al., 2013). The reggie-1-EGFP and reggie-2-EGFP plasmids have been used previously for rescue experiments after reggie-1 siRNA and reggie-2 siRNA-mediated downregulation (Solis et al., 2007; Munderloh et al., 2009; Solis et al., 2013). All plasmids were amplified in *E. coli* and purified by plasmid purification kit. PSD-95-EGFP vector was from Dr. David S. Bredt (Johnson & Johnson) and kindly provided by Dr. Fukata Masaki (National Institute for Physiological Sciences (NIPS), Aichi, Japan).

Transfections occurred in Optifect Transfection Reagent (Life Technologies).

2.5. Stimulation of GluN (NMDAR)-mediated exocytosis of GluA1 (AMPAR1)

LTP was pharmacologically induced in vitro (Ahmad et al., 2012). In brief, medium was removed from coverslips and an extracellular solution (ECS) containing 150 mM NaCl, 2 mM CaCl2, 5 mM KCl, 10 mM HEPES, 30 mM glucose, 0.001 mM tetrodotoxin (TTX), 0.01 mM strychnine and 0.03 mM bicuculline was added (RT). To stimulate NMDA receptors (GluN), samples were incubated for 3 min at RT with ECS containing 300 μM glycine. ECS without glycine served as control. After washes, ECS without glycine was added for 30 min at RT. Neurons were fixed in 4% PFA (PBS, pH 7.3, 15 min, on ice; Jurado et al., 2013), washed with PBS, and blocked in 3% BSA. GluA1 pAb was applied in PBS and 3% BSA to fixed and non-permeabilized cells.

2.6. FM4-64FX uptake

Wt hippocampal neurons, maturated for 8 d in vitro (div) were co-transfected with reggie-1 siRNA or control siRNA together with PSD-95-EGFP, CA-Rab11a-EGFP or EGFP. After 2 d cells were loaded with 10 μM FM4-64FX dye (Invitrogen) in depolarizing buffer (100 mM NaCl, 50 mm KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 15 mM