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Short communication

GSK3 and KIF5 regulate activity-dependent sorting of gephyrin between axons and dendrites

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ABSTRACT

The kinesin KIF5 transports neuronal cargoes into axons and dendrites. Isolated KIF5 motor domains preferentially move into axons, however KIF5 binding to GRIP1 or gephyrin drives the motor into dendrites, to deliver AMPA receptors (AMPARs) or glycine receptors (GlyRs), respectively. At postsynaptic sites, gephyrin forms a multimeric scaffold to anchor GlyRs and $GABA_A$ receptors ($GABA_A$Rs) in apposition to inhibitory presynaptic terminals. Here, we report the unexpected observation that increased intracellular calcium through chronic activation of AMPARs, steers a newly synthesized gephyrin fusion protein (tomato-gephyrin) to axons and interferes with its normal delivery into dendrites of cultured neurons. Axonal gephyrin clusters were not apposed to presynaptic terminals, but colocalized with GlyRs and neuroligin-2 (NLG2). Notably, functional blockade of glycogen synthase kinase-3 (GSK3) and KIF5 normalized gephyrin missorting into the axonal compartment. In contrast, mutagenesis of gephyrin S270, a GSK3 target, did not contribute to axo-dendritic sorting. Our data are consistent with previous observations, which report regulation of kinesin motility through GSK3 activity. They suggest that GSK3 regulates the sorting of GlyR/gephyrin and NLG2 complexes in a KIF5-dependent manner.

Introduction

Gephyrin is a multifunctional protein that binds different cytosolic and transmembrane proteins in neurons (Dumoulin et al., 2009; Kneussel and Betz, 2000; Sassoe-Pognetto and Fritschy, 2000). The majority of gephyrin localizes at postsynaptic sites and clusters inhibitory glycine receptors (GlyRs) and $GABA_A$ receptors (GABARs) in apposition to presynaptic terminals (Kirsch and Betz, 1995; Moss and Smart, 2001). Gephyrin clustering depends on calcium influx through L-type calcium channels, following depolarization (Kirsch and Betz, 1998), whereas the number or size of gephyrin clusters is regulated by neuronal activity (Niwa et al., 2012) and involves the kinases ERK and GSK3 (Tyagarajan et al., 2013), known to act downstream of synaptic transmission (Seo et al., 2007).

At postsynaptic sites, gephyrin has been shown to interact with neuroligin-2 (NLG2), a cell adhesion molecule that mediates transsynaptic interactions with presynaptic neurexins (Poulopoulos et al., 2009). Consistent with a functional interaction of gephyrin and NLG2, neurons lacking the GABARα2 subunit display a loss of gephyrin and NLG2 clustering (Fritschy et al., 2012).

Besides its major function as a postsynaptic organizer, gephyrin is a cargo adaptor that regulates post-Golgi transport, by bridging vesicular GlyRs and the microtubule motor proteins KIF5 and dynein (Maas et al., 2006, 2009).

Little is known about the sorting of newly synthesized gephyrin. In young cultured neurons, gephyrin has been detected in axons (Craig et al., 1996), however at later stages, it is mainly restricted to the somatodendritic compartment (Colin et al., 1996). Application of nocodazole or microinjection of KIF5-specific antibodies interfered with the post-Golgi delivery of gephyrin into dendrites of mature cultured hippocampal neurons (Maas et al., 2009), indicating that gephyrin uses microtubule-dependent kinesin transport to reach the cell periphery. KIF5 motors in general participate in the sorting of different cargo molecules such as synaptic vesicle

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proteins (VAMP2) into axons and neurotransmitter receptors (GluA2) into dendrites (Hirokawa et al., 2010). At the axon initial segment (AIS), a selective filter for axonal entry has been proposed, which depends on the transport efficacy of KIF5 motors and their individual cargoes attached (Song et al., 2009). In fact, axon entry of the dendritic KIF5 cargo GluA2 was much lower, as compared to the axonal KIF5 cargo VAMP2, however whether regulation of sorting and macromolecule filtering depends on changes in neuronal activity, is currently barely understood.

In this study we used a tomato-gephyrin fusion protein to monitor the sorting of newly synthesized protein, immediately after the expression of fluorescent particles. Our data suggest that KIF5 regulates gephyrin sorting by a mechanism that involves GSK3 activity.

Results and discussion

Increased calcium following chronic AMPAR activation reversibly reduces gephyrin clusters in dendrites

In cultured neurons, endogenous gephyrin is mostly associated with the MAP2-positive somato-dendritic compartment from day 5 in vitro (DIV5) onward (Colin et al., 1996) and is mainly localized at subsynaptic and perisynaptic regions (Lorenzo et al., 2004). To study a potential role of neuronal activity and Ca^{2+} increase in the sorting of gephyrin between axons and dendrites, we aimed to use neuronal cultures at later stages. At DIV11, neurons are characterized by spontaneous activity (Bacci et al., 1999), express the classical pre-and postsynaptic markers (Dumoulin et al., 2000) and display differentiated axonal and dendritic compartments (Supplementary Fig. 1A). To circumvent the technical limitation that newly synthesized gephyrin is indistinguishable from formerly synthesized protein at this stage, we expressed a fluorescent gephyrin fusion protein (tomato-gephyrin) and defined the time-point of transfection as 0 min. Time series revealed detectable fluorescent gephyrin signals at about 5 to 7 h after transfection. At 8 h after transfection, tomato-gephyrin formed discrete clusters restricted to the somato-dendritic compartment and colocalized with endogenous gephyrin and the presynaptic vesicle marker SV2 (Supplementary Fig. 1B and C). Expression of the fluorescent tomato-gephyrin fusion protein did not cause a massive overexpression, but led to distinct gephyrin signals, similar as identified by immunostaining of endogenous protein (compare with Fig. 2A, Supplementary Fig. 1B).

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2015.01.005.

Activation of AMPARs had been previously shown to affect the subcellular distribution of gephyrin (Maas et al., 2009). To examine the targeting of newly synthesized tomato-gephyrin molecules upon AMPAR activation, we chronically applied 20 μM AMPA between 2 and 8 h post transfection to the neuronal cultures. AMPA receptors desensitize within milliseconds after ligand binding (Sun et al., 2002), but recover from this depression in the tens of millisecond time range via surface membrane diffusion (Heine et al., 2008). Ca^{2+} imaging with the Ca^{2+} indicator FURA-2 confirmed that this protocol induced an increase in Ca^{2+} levels by about 100% (maximum at 5 min: 146 ± 14.5%, $n = 20$, $p < 0.001$) (Supplementary Fig. 1D and E).

Under control conditions, tomato-gephyrin entered the dendrites and formed typical clusters 8 h post transfection (30.3 ± 2.0 clusters/100 μm neurite, $n = 56$ cells) (Fig. 1A, upper left and B). In contrast, upon AMPAR activation, the number of tomato-gephyrin particles that reached the dendritic periphery were significantly reduced (18.3 ± 1.3 clusters/100 μm, $n = 62$ cells, $p < 0.001$) (Fig. 1A lower left and B) (Maas et al., 2009). This effect was specific, since application of 10 μM of the AMPA receptor antagonist DNQX did not interfere with the sorting and/or delivery of gephyrin fusion protein (26.0 ± 3.4 clusters/100 μm, $n = 61$ cells) (Fig. 1A, upper right and B). Notably, washout of AMPA, followed by a 12 h recovery period, normalized the subcellular distribution of tomato-gephyrin, leading to the typical gephyrin cluster distribution, as detected in dendrites of control cells (control: 30.1 ± 2.1 clusters/100 μm, $n = 22$ cells; washout: 27.7 ± 1.6 clusters/100 μm, $n = 27$ cells) (Fig. 1A lower right and C). These data suggest that increased Ca^{2+} levels, following AMPA receptor activation, temporarily induce downstream activity-dependent mechanisms that interfere either with the sorting or the delivery of somatic gephyrin.

Since gephyrin clustering at postsynaptic sites has been discussed in the context of GSK3 activity (Tyagarajan et al., 2011, 2013), we repeated this assay in the presence of a GSK3 blocker. Application of 2 mM lithium chloride (LiCl) (Beaulieu et al., 2004) partially reversed the effect of AMPA (Fig. 1D and E, control: 34.5 ± 3.2 clusters/100 μm, $n = 8$ cells; AMPA: 19.6 ± 2.1 clusters/100 μm, $n = 10$ cells; LiCl: 26.7 ± 1.8 clusters/100 μm, $n = 12$ cells), similar as seen upon AMPA receptor blockade through DNQX (compare with Fig. 1B). Also 5 μM of the GSK3 inhibitor GSK-IX (Ibrahim et al., 2011) partially normalized the reduced delivery of tomato-gephyrin into the neurite periphery (Fig. 1F and G, control: 26.0 ± 1.0 clusters/100 μm, $n = 10$ cells; AMPA: 15.5 ± 1.1 clusters/100 μm, $n = 10$ cells; GSK-IX: 21.6 ± 1.8 clusters/100 μm, $n = 11$ cells). These data are consistent with GSK3 acting as a downstream kinase, following AMPA receptor activation (Nishimoto et al., 2009) and suggest that this pathway negatively regulates gephyrin targeting.

Chronic AMPAR activation induces missorting of gephyrin into axons

Notably, we made the unexpected observation that increased Ca^{2+} levels, following AMPAR activation not only hindered newly synthesized tomato-gephyrin to enter dendrites (Fig. 2A and B, crossed arrow), but led to prominent clustering along the axon (Fig. 2B, arrowhead).

Axonal gephyrin immunoreactivity has been described in immature cultured hippocampal neurons younger than DIV8, whereas in more mature neurons gephyrin was found to be mainly dendritic (Craig et al., 1996). To distinguish the initial axon from initial dendrites, we immunostained tomato-gephyrin-expressing neurons with an antibody against ankyrin G, an axon initial segment (AIS) marker (Fig. 2C, green channel). Consistent with the literature, quantification of tomato-gephyrin signals of untreated neurons revealed just a very low number of puncta in the AIS of DIV 11/DIV 12 neurons (3.8 ± 0.8 clusters/100 μm, $n = 20$ cells) (Fig. 2C and D).

In contrast, chronic application of 20 μM AMPA increased the number of tomato-gephyrin clusters in axons by more than 500% of control values (20.7 ± 1.9 clusters/100 μm, $n = 39$ cells; $p < 0.0001$) (Fig. 2C and D). Since AMPA was applied prior to the detectable expression of the fluorescent fusion protein, we conclude that axonal tomato-gephyrin clusters were not redistributed from the dendrites, but derive from newly synthesized somatic gephyrin molecules that underwent missorting.

Axonal tomato-gephyrin colocalizes with the inhibitory GlyR and neuroligin 2 at non-synaptic sites

Gephyrin not only mediates synaptic functions, but also regulates non-synaptic processes in molybdenum cofactor (MoCo) biosynthesis (Smolinsky et al., 2008; Stallmeyer et al., 1999) and motor protein transport (Maas et al., 2006, 2009). To assess the nature of axonal gephyrin, we analyzed colocalization of tomato-gephyrin clusters with some postsynaptic gephyrin-binding partners in the presence of AMPA.