DSCR1 is required for both axonal growth cone extension and steering

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Local information processing in the growing cone is essential for correct wiring of the nervous system. As an axon navigates through the developing nervous system, the growth cone responds to extrinsic guidance cues by coordinating axon outgrowth with growth cone steering. It has become increasingly clear that axon extension requires proper actin polymerization dynamics, whereas growth cone steering involves local protein synthesis. However, molecular components integrating these two processes have not been identified. Here, we show that Down syndrome critical region 1 protein (DSCR1) controls axon outgrowth by modulating growth cone actin dynamics through regulation of cofilin activity (phospho/dephospho-cofilin). Additionally, DSCR1 mediates brain-derived neurotrophic factor–induced local protein synthesis and growth cone turning. Our study identifies DSCR1 as a key protein that couples axon growth and pathfinding by dually regulating actin dynamics and local protein synthesis.

Introduction

The ability of an axon to navigate through the developing nervous system depends on the growth cone. In response to extrinsic cues, a growth cone exhibits changes in elongation rate and direction en route to its final destination (Buck and Zheng, 2002; Dent et al., 2011; Jung et al., 2012; Vitirol and Zheng, 2012). Extrinsic cues control growth cone motility through an array of signaling cascades that control actin and microtubule dynamics to regulate growth cone advance and steering (Dent et al., 1999; Schaefer et al., 2002, 2008; Kornack and Giger, 2005; Lowery and Van Vactor, 2009; Vitirol and Zheng, 2012). The regulation of actin polymerization/depolymerization is vital for axon growth and guidance. However, the molecular components mediating this process have not been completely defined. One key protein is cofilin, which regulates axon growth by sequestering and depolymerizing actin filaments. Increasing cofilin activity has been shown to promote neurite extension (Dent et al., 2011), but on the contrary, higher cofilin activity has been associated with growth cone collapse (Aizawa et al., 2001; Hsieh et al., 2006; Piper et al., 2006). Additionally, knockdown of LIM kinases that inactivate cofilin by phosphorylation resulted in inhibition of neurite outgrowth in chick dorsal root ganglion neurons (Endo et al., 2007). These studies thus suggest that cofilin has dual effects on growth cone motility. To reconcile the apparent controversy, it has been proposed that the unique cytosolic environment of a particular growth cone, such as basal actin dynamics and the ratio of cofilin to actin monomer, might determine the effect of cofilin (in)activation on growth cone behavior (Vitirol and Zheng, 2012).

It has also been shown that local synthesis of β-actin in the developing growth cone in response to external stimuli is important for axon guidance and migration (Leung et al., 2006; Yao et al., 2006), and several regulators that mediate local mRNA translation at axonal growth cones, such as the zipcode binding protein 1 (ZBP1), have been identified (Leung et al., 2006; Yao et al., 2006; Willis et al., 2007; Welshhans and Bassell, 2011). Also, brain-derived neurotrophic factor (BDNF) has been shown to induce local β-actin translation during axon development, but the molecular mechanisms involved in local protein synthesis remain to be defined. Despite the identification of numerous proteins that control axonal growth cone development, our knowledge of the components that mediate actin dynamics and axon guidance is not complete.

The Down syndrome critical region 1 protein (DSCR1, also called regulator of calcineurin [RCAN1]) is located on chromosome 21 and is highly expressed in hippocampal neurons (Fuentes et al., 1995). DSCR1 belongs to a conserved family of calcineurin inhibitors called calcipressins, which

*Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; CsA, cyclosporin A; DIV, day in vitro; DSCR1, Down syndrome critical region 1 protein; FMRP, Fragile X mental retardation protein; FRET, Förster resonance energy transfer; G-actin, globular actin; ZBP1, zipper binding protein 1.
includes RCN1P in yeast (Kingsbury and Cunningham, 2000), CBP1 in fungus (Görlich et al., 2000), nebula in Drosophila melanogaster (Chang et al., 2003), and DSCR1 in mouse and human (Casas et al., 2001; Arron et al., 2006). DSCR1 also interacts with Fragile X mental retardation protein (FMRP), an RNA-binding protein that controls mRNA transport and translation, including local translation in dendritic spines (Wang et al., 2012). Absence of FMRP is responsible for Fragile X syndrome (Santoro et al., 2012). It has been suggested that Down syndrome and Fragile X syndrome participate in common biological pathways leading to intellectual disability (Chang et al., 2013). Here we demonstrate a previously unidentified role for DSCR1 in regulating axonal growth cone extension and growth cone turning toward an attractant signal. Our work reveals that DSCR1 regulates the ratio of cofilin and phospho-cofilin to modulate axon outgrowth as well as mediates BDNF-induced local protein synthesis to regulate growth cone turning.

Results

DSCR1 plays an important role in axon development and axonal growth cone steering

In initial studies we demonstrated that DSCR1 is highly expressed in the growth cones of mouse primary hippocampal neurons (Fig. S1, A–E). This suggests that DSCR1 may help regulate axon growth and/or guidance. Immunostaining revealed that wild-type hippocampal neurons at day in vitro are covered by DSCR1 and an axon, which were marked by antibodies against MAP2 and Tau1, respectively (Fig. 1 A). At this time, DSCR1−/− neurons had also extended an identifiable but shorter axon. In contrast, neurons from a DSCR1 transgenic line that overexpresses DSCR1 extended axons that were longer than those from wild-type neurons (Fig. 1, A and B). To more accurately assess the role of DSCR1 in axon development, we monitored axon growth by time-lapse imaging for 12 h starting at DIV 2 (Fig. S1, F and G). Results show that absence of DSCR1 reduces and overexpression of DSCR1 increases the rate of axon growth compared with the rate observed in wild-type control neurons. In addition, the morphologies of DSCR1−/− axons were abnormal, exhibiting ruffling and extensive process elaboration. To determine if DSCR1 also affects growth cone steering, we examined the behaviors of axons of wild-type, DSCR1−/−, and DSCR1 transgenic mice in Dunn chambers in the presence or absence of a gradient of BDNF (Yam et al., 2009; Fig. 1, C and D). Over a 2-h period, we measured the turning angles of individual axonal growth cones (Fig. 1, C–E). The growth cones from control and DSCR1 transgenic mice turned toward the BDNF gradient. DSCR1−/− axons, however, exhibited less growth cone extension and failed to turn toward the source of BDNF (Fig. 1, C–E). Increased levels of DSCR1 also shortened the time required to observe initial turning of a growth cone (Fig. 1 F); turning was observed in growth cones from DSCR1 transgenic mice in ~10 min, whereas ~20 min were required to observe turning by wild-type growth cones. In contrast, growth cones from DSCR1−/− mice did not respond to the BDNF gradient during the entire 2-h long recording period. Together, these results indicate that DSCR1 is required for both axon growth and growth cone steering.

DSCR1 regulates axonal growth cone dynamics by modulating the levels of phospho-cofilin and cofilin

To further examine growth cone extension and steering in axons lacking or overexpressing DSCR1, we performed short-term live imaging of axonal growth cones at DIV 3 and investigated dynamics of filopodia-containing lifeact-GFP (F-actin) and RFP-actin (globular actin [G-actin], F-actin, or total actin; Fig. 2 A). Formation of actin filaments (F-actin) depends on the availability of monomeric G-actin. Ratios of filamentous to G-actin were quantified using fluorescent conjugates of cytochalasin and vitamin D–binding protein, respectively (Van Baalen et al., 1980; Fishkind and Wang, 1993; Lee et al., 2013). Interestingly, compared with those of wild-type neurons, filopodia in growth cones overexpressing DSCR1 had a high F-actin/G-actin ratio and high motility, whereas filopodia of DSCR1-deficient neurons had a low F-actin/G-actin ratio and low motility. Consequently, we hypothesized that DSCR1-dependent modulation of growth cone dynamics results from the alterations of the ratio of F- and G-actin, resulting in fast or slow axon growth. Data presented in Fig. 2 C show that the F-actin level in the leading edge of filopodia of neurons overexpressing DSCR1 was dramatically higher than levels in wild-type and DSCR1−/− growth cones.

Because the activity of the F-actin–severing protein cofilin, which can be assessed by measurement of the cofilin/phospho-cofilin ratio, is crucial for maintaining the balance between F-actin and G-actin, we next compared the levels of cofilin and phospho-cofilin in wild-type, DSCR1-deficient, and DSCR1-overexpressing growth cones to determine whether differences in active (dephospho-)cofilin correlate with the differences in F-actin and G-actin observed above. Indeed, DSCR1−/− growth cones showed significantly higher cofilin/phospho-cofilin ratios than those of wild-type growth cones, and DSCR1-overexpressing growth cones exhibited a lower cofilin/phospho-cofilin ratio, reflecting reduced cofilin activity (Fig. 2, D and E). These findings are consistent with the notion that increased levels of active (dephospho-)cofilin in DSCR1−/− axonal growth cone decrease the F-actin/G-actin ratio, resulting in impaired axonal growth cone extension.

Next, to investigate if cofilin activity indeed regulates axon growth, we attempted to suppress the impact of DSCR1 deficiency on the cofilin/phospho-cofilin ratio in DSCR1−/− growth cones. It is known that DSCR1 inhibits the protein phosphatase calcineurin, which dephosphorylates phospho-cofilin, thereby activating it (Fuentes et al., 2000; Zhou et al., 2004; Wang et al., 2005). In past work, we have shown that DSCR1 regulates dendritic spine morphogenesis by inactivating calcineurin and the level of phospho-cofilin (Wang et al., 2012). Therefore, we first examined if suppression of calcineurin activity by cyclosporin A (CsA) treatment restores the relative levels of cofilin to phospho-cofilin and F-actin/G-actin ratios as well as the length of the DSCR1−/− axons. As expected, we found that treatment of DSCR1−/− neurons with CsA increased the level of phospho-cofilin, but surprisingly reduced the total level of cofilin. Additionally, CsA treatment also restored the F-actin/G-actin ratio to a ratio similar to that in wild-type growth cones (Fig. 3). To further confirm the impact of DSCR1-mediated calcineurin inhibition on axon growth, DSCR1−/− neurons were treated with CsA from DIV 1 until DIV 3 and stained for axonal and dendritic markers. Treatment of DSCR1−/− neurons with CsA clearly promoted axon outgrowth (Fig. 4, A and B), suggesting that DSCR1 mediates axon growth through regulation of calcineurin. To
further verify that DSCR1 plays a key role in axon outgrowth, we compared the effects of CsA on wild-type and DSCR1-overexpressing neurons. We found that treatment of wild-type neurons with CsA reduced the ratio of cofilin/phospho-cofilin and increased both the F-actin/G-actin ratio and mean axonal length (Fig. S2, A, B, and E–G). In contrast, CsA treatment of DSCR1 transgenic neurons did not increase the ratio of cofilin/phospho-cofilin, reduce the F-actin/G-actin ratio, or further reduce the mean length of axons (Fig. S2, C, D, and H–J). Our observation that CsA has little effect on the growth cones and axons of DSCR1-overexpressing neurons argues that calcineurin inhibition by DSCR1 plays a key role in regulating axon growth.
growth cone chemoattractant responsiveness. This result is also consistent with the effect of calcineurin on the cofilin/phospho-cofilin ratio (Fig. 3). Note that wild-type and DSCR1−/− neurons also did not alter phospho-cofilin or cofilin levels after BDNF treatment (Fig. S4, A–D).

To further verify the absence of an impact of the cofilin/phospho-cofilin ratio on the chemoattractant response of growth cones to BDNF, we examined axonal length and growth cone turning in neurons in which the phosphomimetic mutant of cofilin (cofilin S3E) was overexpressed. Overexpression of cofilin
S3E reduces dephosphorylation of endogenous phospho-cofilin through competition with phosphatases, thereby elevating the ratio of phospho-cofilin to cofilin. As expected, cofilin S3E overexpression in DSCR1-deficient neurons resulted in significantly greater axon growth. In contrast, cofilin S3E overexpression did not restore the ability of these growth cones to respond to a chemoattractive gradient of BDNF (Fig. 4, E–G). Consistent with this, overexpression of cofilin S3E in wild-type neurons increased axonal growth but did not appear to further enhance the responsiveness of growth cones to a BDNF gradient (Fig. S3, E–G). Together, these results suggest that DSCR1 can regulate axon outgrowth, but not chemotropic factor responsiveness by influencing the relative levels of phospho-cofilin and cofilin.

**DSCR1 is required for local protein synthesis in axonal growth cone**

Recent data have established an important role for local protein synthesis in the growth cone in the responsiveness of growth cones to chemotropic factors (Brittis et al., 2002; Tcherkezian et al., 2010; Jung et al., 2012). Previously, we showed that the DSCR1 protein is enriched in dendritic spines, and through interaction with the mRNA binding protein FMRP modulates local protein synthesis in dendritic spines, thereby regulating spine morphology (Wang et al., 2012). Consequently, we hypothesized that, similar to its role in spine morphogenesis, DSCR1 may control the responsiveness of growth cones to chemotactants, such as BDNF, through regulation of local protein synthesis. We first determined whether DSCR1 regulates local protein synthesis in growth cones by applying a recently reported technique termed proteins synthesis monitoring. This technique allows for quantitative analysis of protein synthesis and detection of active ribosomes through assessment of occupancy of adjacent sites on ribosomes by tRNAs labeled with Förster resonance energy transfer (FRET) acceptor and donor fluorophores (Barhoom et al., 2011). We transfected primary hippocampal neurons prepared from wild-type, DSCR1−/−, and DSCR1−/− transgenic mice with bulk uncharged tRNA labeled with either Cy3 or rhodamine 110 (Rho110). To assess local protein synthesis, we measured FRET signals between Cy3-tRNA (acceptor) and Rho110-tRNA (donor) after acceptor photobleaching. FRET signals arise when two fluorescent tRNAs occupy adjacent sites on the ribosome, so an increase in the FRET signal reflects active protein synthesis originating from the proximity of FRET pairs on translational machinery (Barhoom et al., 2011). We found that BDNF treatment induced a significant increase in FRET signal in axonal growth cones of DIV 3 neurons from wild-type mice (Fig. 5, A and B). The addition of puromycin, a translational inhibitor, extinguished the BDNF-induced FRET signals (Fig. S4, A and B), indicating that BDNF-induced changes in FRET signals result from local protein synthesis in axonal growth cones. Next, we monitored BDNF-induced local protein synthesis at axonal growth cones of DSCR1-deficient and -overexpressing neurons. No detectable FRET signal was observed after BDNF application in growth cones lacking DSCR1, whereas application of BDNF...
to the growth cones from DSCR1 transgenic mice dramatically increased the FRET signal (Fig. 5, A and B). These data imply that DSCR1 is required for BDNF-dependent local protein synthesis in growth cones. Because local synthesis of β-actin in growth cones has been shown to be essential for chemotactic responses of growth cones to both netrin and BDNF (Leung et al., 2006; Yao et al., 2006), we next examined whether local β-actin mRNA translation is controlled by DSCR1. To this end, we constructed a vector containing the photoswitchable dendra-2 protein fused with the 3′UTR of β-actin mRNA together with two copies of the palmitoylation sequence (dendra2-3′UTR of β-actin), similar to previous studies (Welshhans and Bassell, 2011; Wang et al., 2012). The 3′UTR of the β-actin transcript was sufficient to guide its localization to the axonal growth cone, and newly synthesized dendra-2 protein was monitored after photoconversion and BDNF application. UV light was illuminated on axonal growth cones, which irreversibly converted the green fluorescent dendra-2 to RFP. Newly synthesized green fluorescent dendra-2 proteins in axonal growth cones were quantified from time-lapse images taken every 10 min for 60 min after BDNF application (Fig. 5, C–E). Upon BDNF stimulation, newly synthesized green fluorescent dendra-2 was detected in...
Figure 5. **DSCR1 is required for local protein synthesis in axonal growth cones.** (A and B) Hippocampal primary neurons of wild-type, DSCR1−/−, and DSCR1 transgenic mice were transfected with bulk Cy3-labeled rRNAs and Rho-110-labeled rRNAs. FRET signals are generated when a donor-labeled rRNA is positioned next to an acceptor-labeled rRNA in an active ribosome. Cy3-rRNAs (acceptor) and Rho-110–rRNA (donor) signals were measured both before and after photobleaching to estimate FRET signal intensity. Significantly higher FRET signals were detected in the axonal growth cones when neurons of wild-type and DSCR1 transgenic mice were treated with BDNF, whereas no FRET signal was detected in DSCR1−/− axonal growth cones. Dotted line indicates axonal growth cone and the photobleached area. Pseudo-colored images show the relative fluorescence intensity. Experiments were performed at DIV 3. > 20 axonal growth cones were analyzed for each condition. *, P < 0.05. Values are shown as mean ± SEM and are tested for statistical significance by t test. Bar, 2 µm. (C) Pseudo-colored images of the dendra-2 protein in axon growth cones. Wild-type, DSCR1−/−, and DSCR1 transgenic neurons were transfected with the dendra2-3′ UTR of β-actin vector. Increased intensity of dendra-2 represents local protein synthesis in axonal growth cones. (D and E) Consistent with the results in A, axonal growth cones of DSCR1 transgenic and wild type treated with BDNF showed high fluorescent intensity, suggesting an increase in protein synthesis. DSCR1−/− growth cone, however, showed no local protein synthesis in the same condition. Furthermore, BDNF failed to induce protein synthesis in the presence of a translation inhibitor, anisomycin. Experiments were performed at DIV 3. > 7 axonal growth cones were analyzed for each condition. *, P < 0.0003; **, P < 0.01 compared with wild-type control. Values shown are mean ± SEM and are tested for statistical significance by t test. Bar, 2 µm.
Figure 6. FMRP reduction reduces local protein synthesis in growth cone and prevents axonal growth cone turning in wild-type and DSCR1 transgenic neurons. (A) Wild-type and DSCR1 transgenic neurons were cotransfected with dendra2-β-actin 3′UTR as well as control siRNA or fmr1 siRNA. Increased intensity of dendra-2 represents BDNF-induced local protein synthesis in axonal growth cones. Bar, 2 µm. (B) Axonal growth cones of wild-type and DSCR1 transgenic neurons transfected with control siRNA showed high fluorescent intensity in response to BDNF treatment. In contrast, wild-type neurons transfected with fmr1 siRNA showed no local protein synthesis. Furthermore, DSCR1 transgenic neurons containing fmr1 siRNA failed to translate dendra2-β-actin 3′UTR reporter vector. n > 6 axonal growth cones were analyzed for each condition. *, P < 0.001; **, P < 0.004. (C) Trajectory plots show growth cone turning response of individual neurons of wild-type and DSCR1 transgenic neurons transfected with control siRNA or fmr1 siRNA. n = 10 axonal growth cones were analyzed for each condition. (D) Axonal growth cones of wild-type or DSCR1 transgenic neurons with reduced FMRP failed to turn toward BDNF. *, P < 0.001. Values showed are mean ± SEM and are tested for statistical significance by t test.
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wild-type growth cones, and the increase in fluorescence was abrogated by anisomycin, an inhibitor of translation, suggesting that the BDNF-induced increase in fluorescence resulted from local protein synthesis. In the growth cones of DSCR1−/− neurons, BDNF treatment failed to induce new protein synthesis, whereas in DSCR1 transgenic (DSCR1-overexpressing) growth cones, local protein synthesis was significantly enhanced compared with wild type. It is unlikely that the increase in green fluorescence is caused by diffusion of dendra-2 from the cell body because we confirmed that dendra-2 proteins in the cell body or axon projection do not reach axonal growth cones during the experimental time period (Fig. S4 B). Together, these results indicate that DSCR1 is required for BDNF-induced local β-actin mRNA translation in axonal growth cones.

DSCR1 regulates local β-actin mRNA translation in axonal growth cones through FMRP

To further elucidate how DSCR1 regulates BDNF-induced local β-actin mRNA translation in axonal growth cones, we examined whether DSCR1 controls local protein synthesis through FMRP. Reasons for this are based on previous studies showing (a) FMRP localization at the axonal growth cone (Antar et al., 2006), (b) DSCR1 interacts with FMRP (Wang et al., 2012), and (c) FMRP associates with ZBP1, a protein that affects β-actin mRNA transport as well as translation (Rackham and Brown, 2004; Welshhans and Bassell, 2011). We found that BDNF-dependent β-actin mRNA translation in axonal growth cone was significantly inhibited by fmr1 siRNA transfection into wild-type and DSCR1 transgenic hippocampal primary neurons (Fig. 6, A and B; and Fig. S4 C–E). Additionally, growth cone steering toward BDNF was also significantly inhibited (Fig. 6, C and D). Next, we examined whether DSCR1 controls the phosphorylation status of FMRP in axonal growth cones. Because calcineurin dephosphorylates phosphorylated FMRP upon BDNF treatment (Wang et al., 2012), we measured phosphorylated and dephosphorylated FRMP levels in growth cones of wild-type, DSCR1-deficient, and DSCR1-overexpressing neurons before and after BDNF treatment (Fig. S5). BDNF treatment reduced the levels of phosphorylated FMRP in the growth cones of wild-type and DSCR1-overexpressing neurons, but did not alter the level of phosphorylated FMRP in growth cones lacking DSCR1. This is consistent with our previous findings in dendritic spines because BDNF activates calcineurin via release from DSCR1-mediated inhibition. BDNF application to wild-type and DSCR1-overexpressing growth cones is expected to decrease phospho-FMRP by activating calcineurin, whereas the absence of DSCR1 in DSCR1−/− neurons is expected to be ineffective. Furthermore, we found that dephospho-FMRP is also reduced in axonal growth cones of wild-type and DSCR1 transgenic neurons. This result is consistent with a previous study which showed that dephosphorylation induced rapid degradation of dephospho-FMRP by the ubiquitin-proteasome system (Nalavadi et al., 2012).

Figure 7. A model shows the dual roles of DSCR1 in axonal growth cone. DSCR1 mediates axonal growth cone extension and steering by regulating actin polymerization and local protein synthesis. During axonal growth cone advancement, DSCR1 regulates axon growth by modulating the activity of calcineurin that produces active cofilin to control actin polymerization. However, when axonal growth cone navigates to its target regions responding to signal cues, such as BDNF, DSCR1 becomes a key player in local protein synthesis by changing the status of FMRP phosphorylation, which mediates growth cone turning.
Discussion

In summary, we have shown that DSCR1 plays a crucial role in developing axons by controlling both axon growth and guidance: (a) DSCR1 regulates local cytoskeletal dynamics in the growth cone, which is essential for axon outgrowth; and (b) DSCR1 controls local protein synthesis of β-actin in the growth cone, a process that is crucial for growth cone steering toward BDNF (Fig. 7). In contrast with ZBP1 that mediates local protein synthesis and growth cone turning, but does not alter axon growth (Welshhans and Bassell, 2011), DSCR1 regulates both processes. Axonal growth cone turning is impeded in neurons lacking DSCR1, whereas neurons overexpressing DSCR1 show excessive axonal growth cone turning mediated by increased local protein synthesis, particularly of β-actin. In addition, the axons of neurons lacking DSCR1 display delayed axon development, whereas those of neurons overexpressing DSCR1 extended at a faster rate. Our work reveals that DSCR1 regulates axon outgrowth through its ability to regulate cofilin/phospho-cofilin ratio through calcium-neuron inhibition. In conclusion, our study reveals an important role of DSCR1 in local information processing in axonal growth cones: through regulation of local protein synthesis and cytoskeletal dynamics to control the rate and direction of axon growth. Because DSCR1 has been associated with various clinical manifestations in Down syndrome (Chang et al., 2003, 2013; Chang and Min, 2005; Hoeffer et al., 2007; Dierssen et al., 2011; Wang et al., 2012; Shaw and Chang, 2013), Fragile X syndrome (Wang et al., 2012; Chang et al., 2013), as well as in Alzheimer’s disease (Shaw and Chang, 2013; Shaw et al., 2015), investigating the many roles of DSCR1 in neurons could shed new insights into the cellular and molecular mechanisms underlying developmental defects found in various neurologic disorders.

Materials and methods

Animals

Animals were used in accordance with protocols approved by the Animal Care and Use Committees of the Ulsan National Institute of Science and Technology. DSCR1+/− and DSCR1 transgenic mice were obtained from K. Baek (Sungkyunkwan University, Seoul, South Korea). C57BL/6 mouse strain was purchased from Hyochang Science.

Cell culture

Throughout this paper hippocampal primary neurons at DIV 2 or 3 were used as indicated. Hippocampal neuronal culture was prepared as previously described (Wang et al., 2012). In brief, hippocampi were dissected from E18 mouse embryos, followed by trypsin treatment. A 24-well plate containing 12-mm glass coverslips or 6-well plate containing 18-mm square glass coverslips (In Vitro Scientific) coated with poly-d-lysine (50 µg/ml) were used for seeding neurons. Lipofectamine 2000 (Invitrogen) and Interferin (Polyplus Transfection) were used to transfected neurons with DNA and rhodamine 110 (Rho110)/Cy3-labeled tRNA, respectively.

Plasmids constructions and preparation

Two repeats of GAP-43 palmitoylation sequence and the mouse β-actin 3’UTR were inserted into the pDendra2-C vector (Evrogen) to create pPali2-Dendra2-β-actin 3’UTR. Lite-actin-EGFP vector was purchased from Ibidi. pEGFP-cofilin plasmid was purchased from Addgene, and Fmr1 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc.

Immunocytochemistry

Hippocampal primary neurons were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in cytoskeleton buffer (10 mM MES, pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl2) for 15 min at 37°C, and then treated twice with freshly prepared 0.1% (wt/vol) sodium borohydride for 10 min to reduce background fluorescence. Next, PBS washing was done for 10 min at RT, followed by 0.2% Triton X-100 in PBS for 10 min. Neurons were then blocked for 1 h at RT with PBS containing 1% BSA. Immunostaining was performed by applying primary antibodies as indicated (overnight at 4°C), followed by addition of Alexa Fluor–conjugated secondary antibodies (1:2,000; Invitrogen) for 1 h at RT. Images were taken with LSM780 (confocal; ZEISS) or Elyra S.1 (structured illumination microscopy; ZEISS). Primary antibodies used in this paper are as follows: DSCR1 polyclonal antibody (1:250; Abgent), MAP2 polyclonal antibody (1:10,000; Abcam), tau-1 monoclonal antibody (1:500; EMD Millipore), β-tubulin monoclonal antibody (1:500; Sigma-Aldrich), cofilin antibody (1:150; Abcam), and phospho-cofilin antibody (1:150; Abcam). We also used Alexa Fluor 488– or 555–conjugated Phalloidin dye to label actin filaments.

Monomeric G-actin and actin filaments staining and analysis

Hippocampal primary neurons were fixed and washed as described in the Immunocytochemistry section. After permeabilization and blocking, neurons were incubated with DBP (10 µg/ml; EMD Millipore) in PBS at RT for 1 h, followed by PBS washing and anti-DBP antibody (Dako) incubation. Images were acquired with Elyra S.1 (structured illumination microscopy) and then processed and analyzed using ZEN (ZEISS), ImageJ, and Metamorph (Molecular Devices) software. Ratio images were generated using Metamorph software as described previously (Lee et al., 2013). Profiles of the F-actin/G-actin ratio in filopodia was obtained and analyzed by drawing lines from the leading edge to the adjacency center zone of the growth cone and then averaged.

Axon growth cone steering assay using Dunn chamber

Hippocampal neurons were plated on 18-mm square coverslips (Thermo Fisher Scientific), and the BDNF gradient was established by adding it to the outer well of the Dunn chamber. Images were taken every 5 min for 2 h. Dunn chamber axon guidance assays and analyses were performed as reported previously (Yam et al., 2009). During Dunn chamber assay, 5 µM CsA was added as indicated.

Long-term live imaging

Hippocampal neurons isolated from wild-type, DSCR1−/−, and DSCR1 transgenic mice were transfected with EGFP or RFP-actin and lifeact-GFP to monitor the growth of axons. Live cell imaging was performed in an environmental chamber maintaining 37°C and 5% CO2 throughout the experiment. Images were taken every hour for 12 h (for EGFP) or every 5 s for 10 min (for RFP-actin and lifeact-GFP) using a confocal microscope (LSM780; ZEISS) with a 40X oil objective (NA 1.3, Plan Apo; Nikon) at 5-s intervals for 10 min. Superresolution microscope images

Structured illumination microscopy (Elyra S.1; ZEISS) was used to obtain super-resolution images. For structured illumination microscopy images, hippocampal neurons were fixed and treated with sodium borohydride and stained with antibodies as described in the Immunocytochemistry section. 1.21-µm Z-stacks of high-resolution image frames were collected in three rotations with 488-, 561-, and 633-nm lasers. Images were then reconstructed using the ZEN software.
tRNA FRET assay
Total yeast tRNAs, obtained from Anima Biotech, were labeled at dihydrouridine positions with either Rhodamine or Cy3, as previously described (Barhourom et al., 2011). To yield fluorescent-labeled tRNAs, DIV 2 neurons were transfected with fluorescent-labeled tRNAs labeled with Rhodamine or Cy3 using Interferin (Polyplus Transfection), and FRET assay was performed on DIV 3 neurons. FRET signals were measured after 1 h of BDNF treatment. Neurons transfected with tRNAs were treated with BDNF in the presence or absence of puromycin (0.5 mM; Cayman Chemical) for 1 h. Next, acceptor photobleaching experiment was performed for FRET efficiency analysis by using the LSM780 confocal microscope. The red fluorescence (561 nm) of the acceptor was bleached in axonal growth cones, and the intensity of donor green fluorescence (488 nm) was detected before and after photobleaching of the acceptor. FRET efficiency was determined by using the FRET acceptor bleaching analysis module in the ZEN imaging software, using the following equation:

\[
\text{Efficiency (\%)} = \frac{D_{\text{Pre}} - D_{\text{Post}}}{D_{\text{Pre}}} \times 100.
\]

\(D_{\text{Pre}}\) represents donor fluorescent intensity of the region before bleaching and \(D_{\text{Post}}\) shows the fluorescent intensity of the donor in the post-bleaching image. Note that only a subpopulation of active ribosomes will harbor a donor (acceptor FRET tRNA pair), as other configurations (e.g., wild type/donor, donor/donor, etc.) occur in parallel.

Local mRNA translation assay
Cultured hippocampal neurons were transfected with pPalx2-Dendra2-β-actin 3′UTR reporter at DIV 2, and the translational assay was performed 24 h after the transfection, similar to previous work (Wang et al., 2012). In brief, neurons were exposed to human BDNF (30 ng/ml; Almond Labs) or translation inhibitor, anisomycin (10 µM; Cayman Chemical) for 1 h. Next, acceptor photobleaching experiment was performed for FRET efficiency analysis by using the LSM780 confocal microscope. The red fluorescence (561 nm) of the acceptor was bleached in axonal growth cones, and the intensity of donor green fluorescence (488 nm) was detected before and after photobleaching of the acceptor. FRET efficiency was determined by using the FRET acceptor bleaching analysis module in the ZEN imaging software, using the following equation:

\[
\text{Efficiency (\%)} = \frac{D_{\text{Pre}} - D_{\text{Post}}}{D_{\text{Pre}}} \times 100.
\]

\(D_{\text{Pre}}\) represents donor fluorescent intensity of the region before bleaching and \(D_{\text{Post}}\) shows the fluorescent intensity of the donor in the post-bleaching image. Note that only a subpopulation of active ribosomes will harbor a donor (acceptor FRET tRNA pair), as other configurations (e.g., wild type/donor, donor/donor, etc.) occur in parallel.

Statistical analysis
Statistical values shown are mean ± SEM. Statistical significance was measured by t test using Prism 5.0 software (GraphPad Software).

Online supplemental material
Fig. S1 shows that DSCR1 is present in the axonal growth cone. Fig. S2 shows the effect of Ca²⁺ on the level of phospho-cofilin and phospho-cofilin in wild-type and DSCR1 transgenic growth cone filopodia. Fig. S3 shows the effect of BDNF on the level of phospho-cofilin and cofilin in axonal growth cones of wild-type and DSCR1−/− neurons and the effect of wild-type cofilin and cofilin S1E overexpression on the ratio of cofilin/phospho-cofilin in wild-type neurons. Fig. S4 shows axonal growth cone turning in wild-type and DSCR1 transgenic neurons transfected with fmr1 siRNA. Fig. S5 shows the effect of BDNF on the level of phospho-FMRP and FMRP in axonal growth cones of wild-type, DSCR1−/−, and DSCR1 transgenic neurons. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201510107/DC1.

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