Cellular/Molecular

The Schizophrenia Susceptibility Gene *Dysbindin* Regulates Dendritic Spine Dynamics

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Dysbindin is a schizophrenia susceptibility gene required for the development of dendritic spines. The expression of dysbindin proteins is decreased in the brains of schizophrenia patients, and neurons in mice carrying a deletion in the *dysbindin* gene have fewer dendritic spines. Hence, dysbindin might contribute to the spine pathology of schizophrenia, which manifests as a decrease in the number of dendritic spines. The development of dendritic spines is a dynamic process involving formation, retraction, and transformation of dendritic protrusions. It has yet to be determined whether dysbindin regulates the dynamics of dendritic protrusions. Here we address this question using time-lapse imaging in hippocampal neurons. Our results show that dysbindin is required to stabilize dendritic protrusions. In dysbindin-null neurons, dendritic protrusions are hyperactive in formation, retraction, and conversion between different types of protrusions. We further show that CaMKIIα is required for the stabilization of mushroom/thin spines, and that the hyperactivity of dendritic protrusions in dysbindin-null neurons is attributed in part to decreased CaMKIIα activity resulting from increased inhibition of CaMKIIα by Abi1. These findings elucidate the function of dysbindin in the dynamic morphogenesis of dendritic protrusions, and reveal the essential roles of dysbindin and CaMKIIα in the stabilization of dendritic protrusions during neuronal development.

Key words: CaMKIIa; dendritic spine; dynamics; dysbindin; hippocampus; schizophrenia

Introduction

Dysbindin is a coiled-coil domain containing protein, initially discovered as a dystrophin-binding protein and later found to be one of eight subunits of biogenesis of lysosome-related organelles complex 1 (BLOC-1; Benson et al., 2001; Li et al., 2003). Single-nucleotide polymorphisms of the *dysbindin* gene (*Dtnbp1*) have been associated with higher risk for schizophrenia, and the postmortem brains of schizophrenia patients consistently exhibit low levels of dysbindin proteins and mRNAs (Straub et al., 2002; Talbot et al., 2004; Weickert et al., 2008). Mice carrying a deletion mutation in *Dtnbp1* (*sdy* mice, express no dysbindin proteins) have more of the cell-surface dopamine D2 receptors, which have long been targeted in the treatment of schizophrenia (Howes and Kapur, 2009; Ji et al., 2009).

Proper synaptic connections between neurons are critical for brain function. In schizophrenia patients, however, MRI studies show that functional connectivity between brain regions is impaired, and there are fewer dendritic spines in their prefrontal cortex and hippocampus (Garey et al., 1998; Glantz and Lewis, 2000; Kolluri et al., 2005; Kolomeets et al., 2005; Lynall et al., 2010). Therefore, it is thought that neuronal dysconnectivity underlies anomalous brain function associated with schizophrenia. Our recent study suggests that dysbindin contribute to the establishment of neuronal connectivity by regulating the development of dendritic protrusions (Jia et al., 2013), including dendritic spines (tiny dendritic protrusions where excitatory synapses are formed) and filopodia (long, thin protrusions that predominant in young neurons; Wenzel and Bogolepov, 1976; Li and Sheng, 2003). Hippocampal neurons of *sdy* mice have fewer dendritic spines and more filopodia, and synaptic connectivity within the entorhinal cortex-hippocampus circuit is miswired (Jia et al., 2013).

The development of dendritic protrusions is a dynamic process involving addition of new protrusions, retraction of existing ones, and conversion between one type of protrusions (stubby spines, mushroom spines, thin spines, filopodia) to another (Parnass et al., 2000). These dynamic events in the morphogenesis of dendritic protrusions facilitate not only formation and maturation, but also plasticity of synaptic connections, which are needed to establish and refine neural circuits. It remains to be determined, however, whether dysbindin regulates the dynamic changes of dendritic protrusions during development.

Here by using time-lapse imaging, we found that hippocampal neurons of *sdy* mice are hyperactive in addition, retraction and transformation of dendritic protrusions. Investigating mechanisms might account for this hyperactivity, we found that the calcium/calmodulin-dependent protein kinase CaMKIIα is required to stabilize dendritic protrusions, and that decreased CaMKIIα activity in *sdy* mice contributes to the hyperactivity of dendritic protrusions. We also demonstrated that diminished CaMKIIα activity is caused by enhanced interaction of CaMKIIα and Abi1, a protein forming complexes with dysbindin. This study reveals a key mechanism by
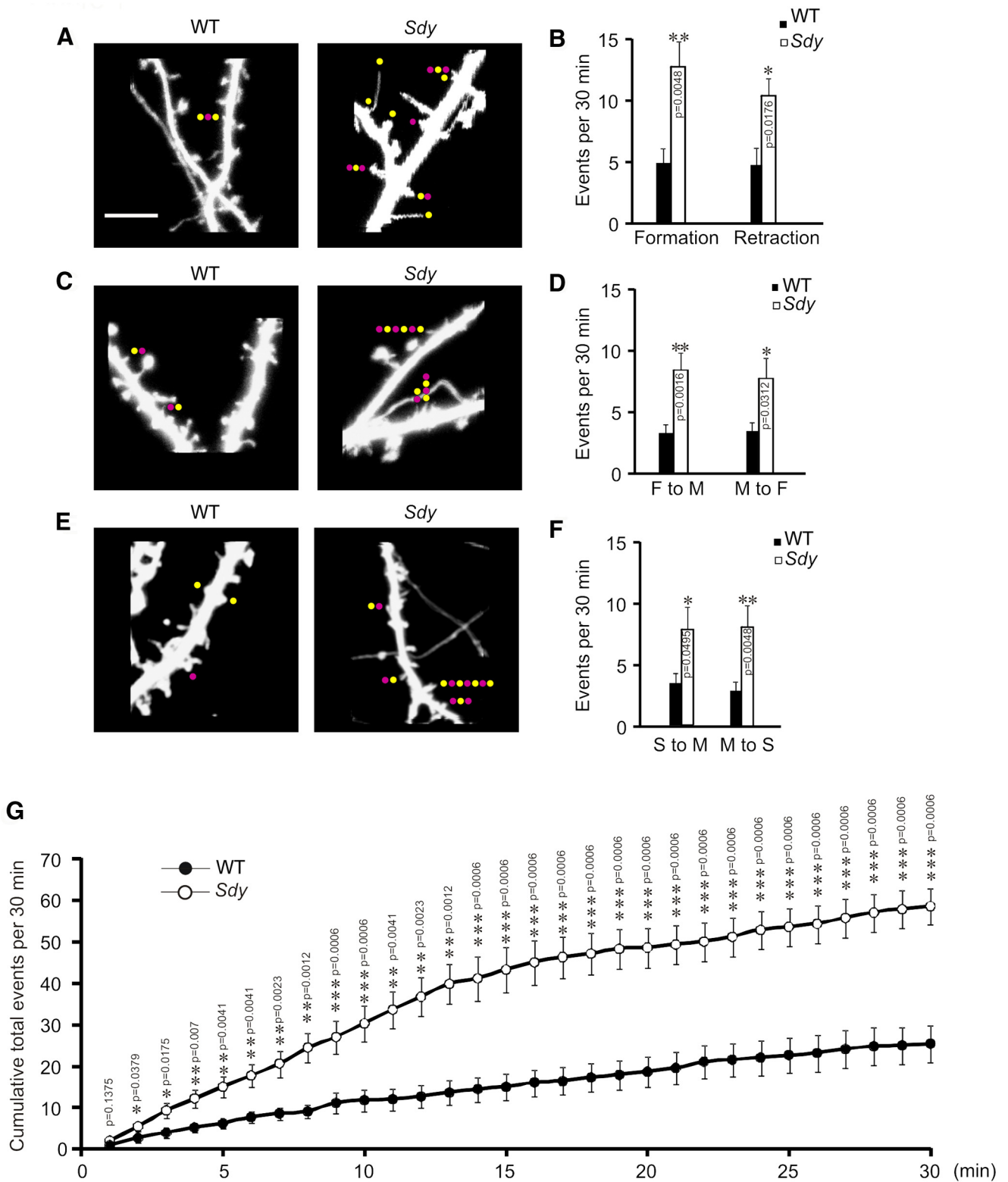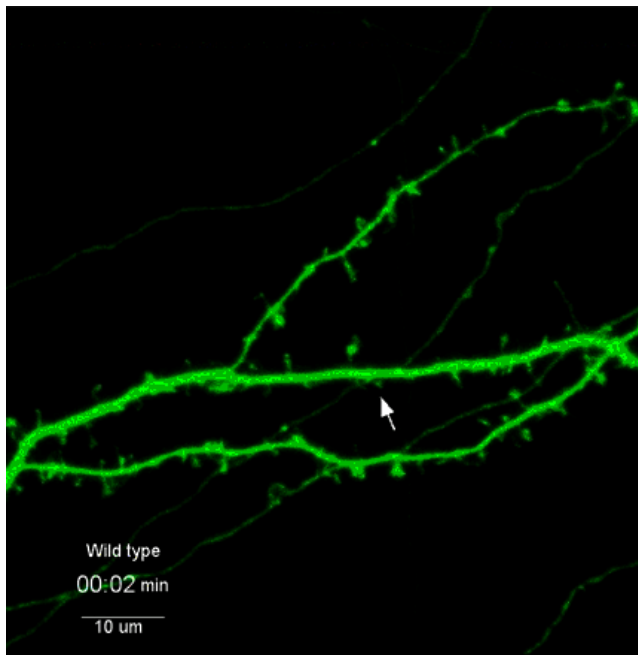
Figure 1. The dynamics of dendritic protrusions are increased in *sdy* neurons. Hippocampal neurons from wild-type or *sdy* mice were transfected at 14 DIV with the Venus construct and imaged every minute for 30 min at 17 DIV. ***A***, ***C***, ***E***, Representative images superimposed from those taken at 4 adjacent time points during the 30 min live-imaging period. In ***A***, red dots represent protrusion retraction, yellow dots indicate protrusion addition; in ***C***, red dots represent the transformation from mushroom/thin spines to filopodia, yellow dots indicate the transformation in the opposite direction; in ***E***, red dots represent the conversion from mushroom/thin to stubby spines, yellow dots indicate the conversion in the opposite direction. ***B***, ***D***, ***F***, Quantification of ***A***, ***C***, ***E***. ***G***, Cumulative frequency plot of total dynamic events occurring during the 30 min imaging period. Scale bar, 5 μm. Data are presented as mean $\pm$ SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Movie 1. Dynamics of dendrite protrusions in wild-type neurons. Hippocampal neurons from wild-type mice were transfected with the Venus construct at 14 DIV. This movie shows confocal images of transfected dendrites taken every minute for 30 min at 17 DIV.

Movie 2. Dynamics of dendrite protrusions in *sdy* neurons. Hippocampal neurons from *sdy* mice were transfected with the Venus construct at 14 DIV. This movie shows confocal images of transfected dendrites taken every minute for 30 min at 17 DIV.

which dysbindin regulates the development of dendritic spines and an essential role of CaMKIIα in the dynamics of dendritic protrusions.

Materials and Methods

Animals, plasmids, and reagents. All animal procedures followed the NIH Guidelines *Using Animals in Intramural Research*, and were approved by the National Institute of Mental Health Animal Care and Use Committee. Four mice of the same sex were housed in one cage in a room with a 12 h light/dark cycle. *Sdy* mice (backcrossed with the C57BL/6J mice for >10 generations) were purchased from The Jackson Laboratory. Littermates of wild-type and *sdy* mice (of either sex) were used in quantitative reverse-transcription (RT)-PCR and immunoblotting analyses. SiRNAs targeting mRNAs encoding dysbindin and scrambled oligonucleotides were inserted into the pSuper plasmid. The sequences of dysbindin siRNAs and scrambled oligonucleotides are as follows: GCACCACTCTACCCTATTA (dys-siRNA1), CGGC-CTTGGTCACATTAAA (dys-siRNA2), GACGTTAAGCGACAAGTCA (dys-siRNA3), and GACGTGAACGGATAACACT (scrambled oligonucleotides). The CaMKII siRNA was purchased from Thermo Fisher Scientific (catalog #A-059173-14). Antibodies against the following proteins were obtained commercially: phosphorylated CaMKIIα (1: 500; catalog #3361, Cell Signaling Technology), CaMKIIα (1:1000; catalog #13-7300, Invitrogen), Abi1 (catalog #D147-3, MBL), actin (1:10,000; catalog #A4700, Sigma-Aldrich), and IgG (H+L; HRP-conjugated; Bio-Rad). A rabbit polyclonal anti-dysbindin antibody was raised (Covance) against the C-terminal region of rat dysbindin (amino acids 202–350) and affinity-purified before use. Immunoblots were analyzed using ImageJ software.

Neuronal culture and transfection. Cultures of hippocampal neurons were prepared from embryonic day (E)18–19 rat or mouse embryos (of either sex) as previously described (Jia et al., 2013) and grown in neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% glutamax (Invitrogen). Hippocampal neurons were seeded at a density of 300 cells/mm^2. Neurons were transfected with Lipofectamine 2000 (Invitorgen, for plasmids) or DharmaFECT (Thermo Scientific, catalog #T-2010-01, for siRNA oligonucleotides).

Time-lapse imaging and analysis of dendritic protrusions. Coverslips seeded with hippocampal neurons were placed in a chamber perfused with artificial CSF (ACSF), consisting of the following (in mM): 124 NaCl, 26 NaHCO₃, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$ 1.3 MgSO$_4$, and 10 D-glucose. ACSF was maintained at a temperature of 30°C. We used an Olympus FV1000 confocal microscope with a 60× (NA 1.35) objective for time-lapse imaging and a Zeiss LSM510 confocal microscope (63× objective, NA1.4) for imaging cells fixed in PBS containing 4% formaldehyde and 4% sucrose. Images were collapsed into 2D projections and analyzed with the MetaMorph software (Molecular Devices) for the number and dimension of dendritic protrusions. Images taken from adjacent time points were compared with track the shape change, appearance, or disappearance of dendritic protrusions. We divided all dendritic spines (0.5– 6 μm in length) into two subgroups: mushroom/thin spines (with an enlarged head and constricted neck) and stubby spines (without constricted necks). Long, pointed dendritic protrusions that are 3–6 μm long were categorized as filopodia. Formation of protrusions was defined as generation of new spines or filopodia, retraction was disappearance of pre-existing spines or filopodia, and conversion was the transformation of spines and filopodia into other types of protrusions. These dynamic events were analyzed by comparing consecutive images taken every minute during a 30 min period.

RT-PCR. At 3 weeks of age, the hippocampus of *sdy* mice and their wild-type littermates (of either sex) were lysed in Trizol (Invitrogen). mRNAs were extracted and transcribed into cDNAs with the oligo (dT) 20 primer. The following primers (CaMKIIα: 5′ primer AAGCACCCATGGATCTCG-CACC, 3′ primer GGCTTCGATCAGCTGCTCTGTCAC; dysbindin: 5′ primer CAGGACGTGGAGTCCTTGAT, 3′ primer GCATGGGCCTGTT-TATGTCT; actin: 5′ primer GCTCCTCCTGAGCGCAAGTACTC, 3′ primer CTCATCGTACTCCTGCTTGCTG) were used for real-time PCR.

Western blotting and antibodies. At 2–3 weeks of age, the hippocampus of *sdy* mice and their wild-type littermates (of either sex) was lysed in RIPA buffer (20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail). The cell lysate was electrophoresed through SDS-PAGE gels, transferred to PVDF membranes, and subjected to immunoblotting with antibodies against phosphorylated-CaMKIIα (1:500; catalog #3361, Cell Signaling Technology), CaMKIIα (1:1000; catalog #13-7300, Invitrogen) and actin (1:10,000; catalog #A4700, Sigma-Aldrich). IgG(H+L)-HRP-conjugated secondary antibodies were obtained from Bio-Rad. Immunoblots were quantified using ImageJ software.

Immunocytochemistry. Hippocampal neurons grown on coverslips were fixed with PBS containing 4% formaldehyde and 4% sucrose for 12 min.
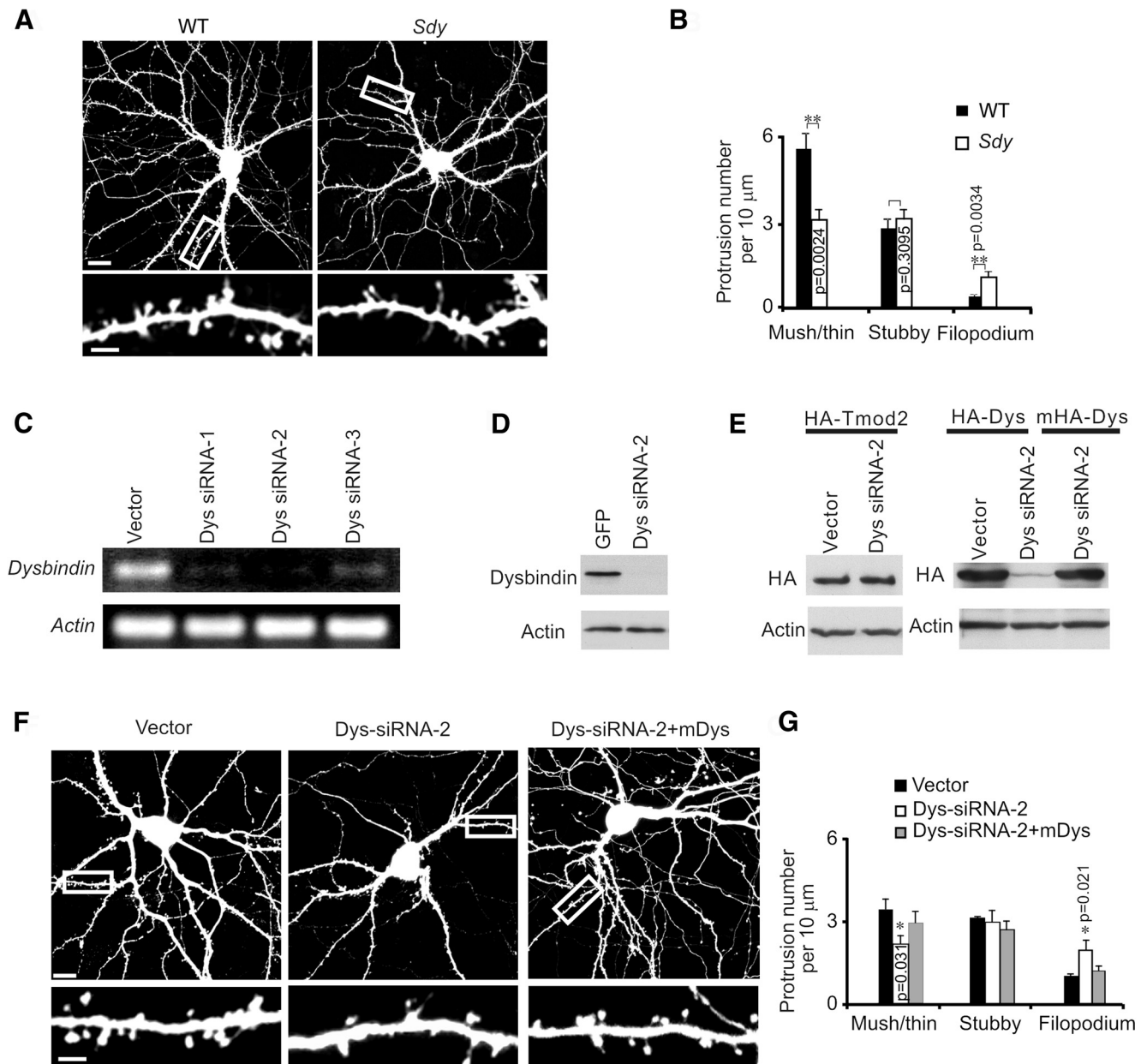
Figure 2. The change to dendritic protrusion density in *sdy* and dysbindin knockdown neurons. Cultured hippocampal neurons were prepared from *sdy* mice or their wild-type littermates (***A***, ***B***) or rats (***F***, ***G***), and transfected with the Venus construct alone (***A***, ***B***) or along with lentivirus expressing dysbindin siRNA and siRNA-resistant dysbindin (mDys) at 14 DIV. ***A***, ***F***, Representative images of neurons and dendrites. Scale bars: neurons, 20 μm; dendrites, 5 μm. ***B***, ***G***, Quantification for ***A*** and ***F***; $n = 15$ cells in each group; data are presented as mean $\pm$ SEM; *$p < 0.05$, **$p < 0.01$. ***C***, ***E***, Cultured wild-type cortical neurons were infected with lentivirus expressing EGFP alone or along with siRNAs targeting dysbindin mRNAs at 5 DIV and harvested for RT-PCR and immunoblotting analysis for endogenous dysbindin mRNAs and proteins at 2 weeks after infection. ***D***, HEK 293 cells were transfected with plasmids expressing HA-tagged dysbindin, tropomodulin 2- (Tmod2, containing no dysbindin siRNA-2 binding sites) or siRNA-resistant dysbindin along with the dysbindin siRNA construct, and harvested at 2 d after transfection for immunoblotting.

After rinse, cells were incubated with the anti-CaMKIIα or MAP2 (1:200; Sigma-Aldrich, catalog #M9942) antibody overnight at 4°C, and then with AlexaFluor 555 secondary antibodies for 2 h at room temperature.

Immunoprecipitation. The P2 (crude synaptosomal) fraction was prepared as described previously (Lau et al., 1996). Briefly, hippocampal tissue from mice of either sex was homogenized in ice-cold homogenization buffer supplemented with protease and phosphatase inhibitors (Roche) using a glass dounce homogenizer. Nuclear fraction was removed via centrifugation (800 × g for 10 min at 4°C). The P2 fraction was isolated by centrifugation at 10,000 × g for 15 min at 4°C, and solubilized in a nondenaturing lysis buffer (1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 20 mM Tris HCl, pH 8, supplemented with protease and phosphatase inhibitors) at 4°C for 1 h. Synaptosomal proteins (500 μg) were incubated with Protein G Sepharose Fast Flow 4 beads (GE) conjugated to anti-Abi1 monoclonal Abs (catalog

#D147-3, MBL) or purified rabbit IgG Ab as an IP control. After wash with the lysis buffer, bound proteins were eluted by boiling and separated by gel electrophoresis on 12% acrylamide gels for immunoblotting. Quantification of immunoblotting was based on three independent experiments.

Results

Dysbindin regulates the dynamics of dendritic protrusions

To determine whether dysbindin regulates the dynamics of synaptic connections during development, we used time-lapse imaging to examine dendritic protrusions (including mushroom spines, thin spines, stubby spines, and filopodia) in cultured hippocampal neurons taken from *sdy* and wild-type mice. Because of the limited spatial resolution of confocal microscopy, we classi-
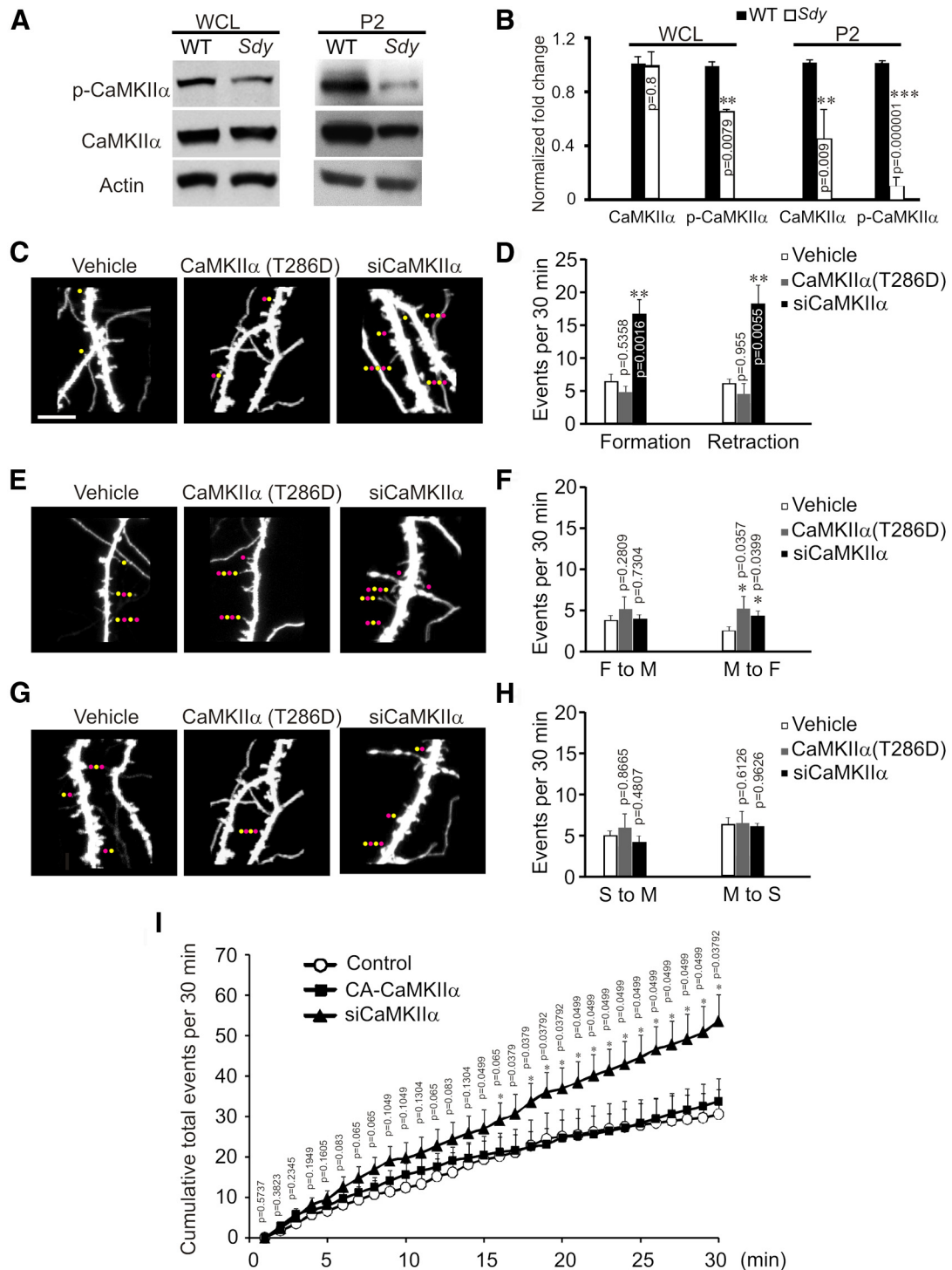
Figure 3. CaMKIIα is required for the stabilization of dendritic protrusions. **A, B**, Hippocampi of 2- to 3-week-old *sdy* mice and their wild-type littermates were removed for analyses of CaMKIIα and CaMKIIα phosphorylated at Thr286 in whole-cell lysates (WCL) and the P2 fraction. **C–I**, Hippocampal neurons were transfected at 14 DIV with the Venus construct along with the CA-CaMKIIα or the CaMKIIα siRNA oligonucleotides, and imaged every minute for 30 min at 17 DIV. **A**, Representative blots. **B**, Quantification for **A**; $n = 4$ experiments. **C, E, G**, Representative images superimposed from those taken at 4 contiguous time points during the 30 min live-imaging period. Red dots represent protrusion retraction (**C**), the transformation from mushroom/thin spines to filopodia (**E**), and the conversion from mushroom/thin to stubby spines (**G**); yellow dots indicate the protrusion addition (**C**), the transformation from filopodia to mushroom/thin spines (**E**), and the conversion from stubby to mushroom/thin spines (**G**). **D, F, H**, Quantification of **C, E, G. I**, Cumulative frequency plot for the total number of all types of dynamic events taking place during the 30 min period. Scale bar, 5 μm. Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$.

fied mushroom and thin spines into one group (mushroom/thin spines). Neurons were transfected at 14 d *in vitro* (DIV) with a construct expressing Venus (a mutant of yellow fluorescent protein) and then imaged at 16–21 DIV, when dendritic protrusions

undergo dynamic formation, retraction, and transformation (Parnass et al., 2000; Nagai et al., 2002). We imaged the same dendritic branch (secondary and associated tertiary dendrites) every minute for 30 min, and analyzed dynamic events taking place

during the imaging period, including formation of new protrusions, retraction of existing protrusions, and conversion between mushroom/thin and stubby spines, between mushroom/thin spines and filopodia, and between stubby spines and filopodia. We found that in 16–21 DIV neurons, the frequency of conversion between stubby spines and filopodia was very low (<1 event in 30 min), and therefore focused our analysis on other types of dynamic changes. All analyzed activities were more dynamic in *sdy* than in wild-type neurons (Fig. 1*A*–*F*; Movies 1, 2). During the imaging period, consequently, protrusions on *sdy* neurons underwent more total dynamic changes than those on wild-type neurons (Fig. 1*G*). These results indicate that the loss of dysbindin leads to an enhancement in the dynamics of dendritic protrusions, thereby reducing the stability of synaptic connections.

This hyperactivity in the morphogenesis of dendritic protrusions might further result in changing their overall numbers. To assess protrusion number, we transfected hippocampal neurons from *sdy* or wild-type mice with the Venus construct at 14 DIV, and fixed them at 17 DIV. Compared with wild-type neurons, *sdy* neurons had more filopodia and fewer mushroom/thin spines (Fig. 2*A*,*B*). The changes in filopodia and mushroom/thin spines can result from imbalanced formation and retraction of protrusions. Indeed, we noticed that in *sdy* neurons although the total amounts of formation and retraction for all three types of protrusions (mushroom/thin spines, stubby spines and filopodia) were comparable, newly added mushroom/thin spines outnumbered retracted ones (formation 1.94 ± 0.45 vs retraction 2.27 ± 0.57), whereas more filopodia were added than retracted (formation 2.55 ± 0.49 vs retraction 1.32 ± 0.37) during the 30 min imaging period. In wild-type neurons, however, all three types of protrusions undergo balanced formation and retraction during the 30 min imaging period.

To test whether this change in protrusion number is due to cell-autonomous effects of dysbindin, we generated constructs expressing siRNAs against dysbindin and tested their specificity and efficacy against both endogenous and overexpressed dysbindin (Fig. 2*C*–*E*). The dys-siRNA2 (one of the effective and specific siRNAs) and the Venus construct were cotransfected into cultured hippocampal neurons (14 DIV). Neurons transfected with the dys-siRNA construct had fewer mushroom/thin spines and more filopodia than control cells (Fig. 2*F*,*G*). These effects, however, were abolished when the dys-siRNA was cotransfected with a construct expressing dysbindin harboring silent mutations in the siRNA binding region (thereby resistant to dys-siRNA; Fig. 2*E*–*G*). Because of our low transfection efficiency (<0.05%), the changes to dys-siRNA-
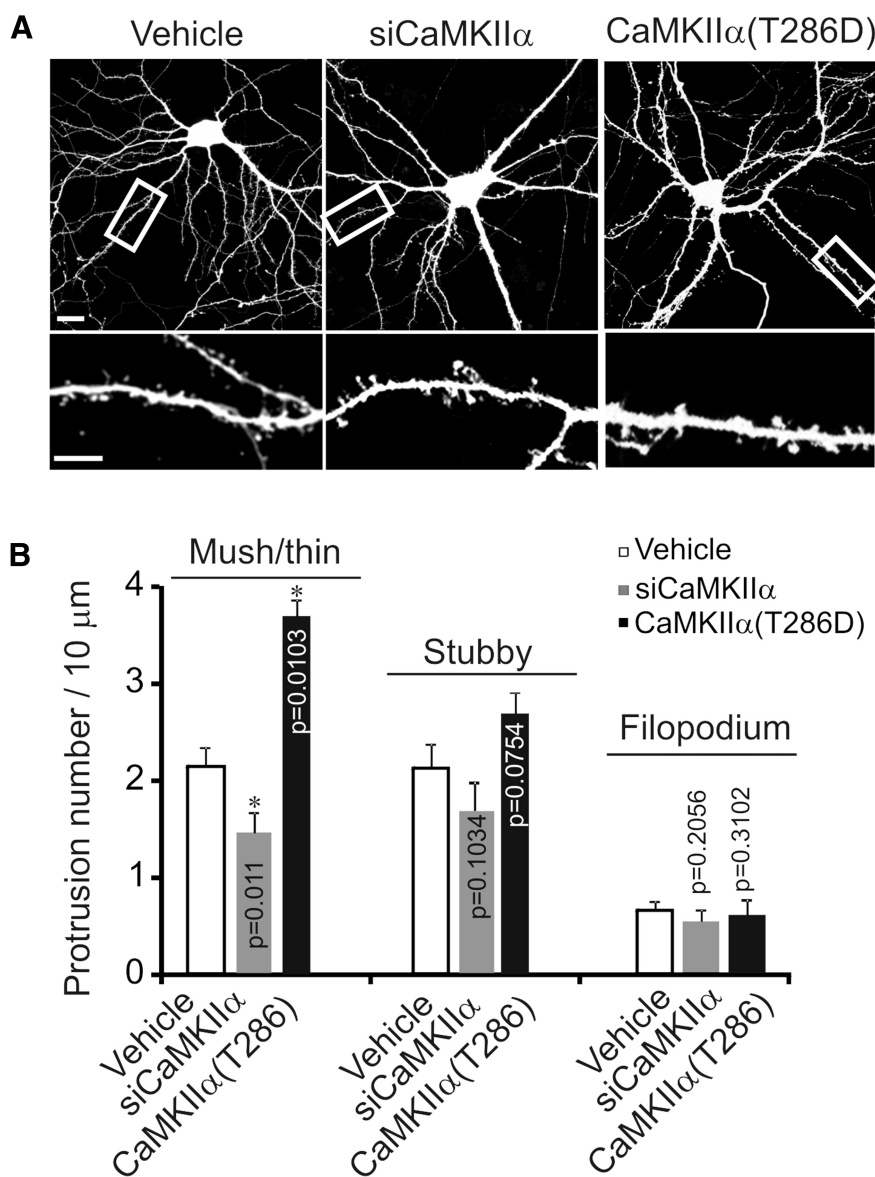

Figure 4. CaMKIIα controls the number of mushroom/thin spines. Cultured hippocampal neurons from rats were transfected at 14 DIV with indicated plasmids. *A*, Representative images of neurons (top) and dendrites (bottom). *B*, Quantification for A, *n* = 15 neurons for each group. Data are presented as mean ± SEM; **p* < 0.05. Scale bars: neurons, 20 μm; dendrites, 5 μm.

transfected neurons are likely induced by cell-autonomous effects of dysbindin knockdown.

Together, these results indicate that dysbindin regulates the dynamics and composition of dendritic protrusions.

CaMKIIα is required to stabilize dendritic protrusions
Having found that dysbindin regulates the dynamics of dendritic protrusions, we went on to investigate the underlying mechanism. Because CaMKII activity is required for experience-dependent stabilization of dendritic spines, and the expression of CaMKII is decreased in the prefrontal cortex of *sdy* mice (Wilbrecht et al., 2010; Papaleo et al., 2012), we tested whether CaMKII contributes to the regulation of dendritic protrusions by dysbindin. We first examined whether CaMKIIα expression is also changed in the hippocampus of *sdy* mice. We prepared whole-cell lysates from the hippocampus of *sdy* mice and their wild-type littermates at the age of 2–3 weeks. Analyzing by immunoblotting, we found that in *sdy* mice although the protein
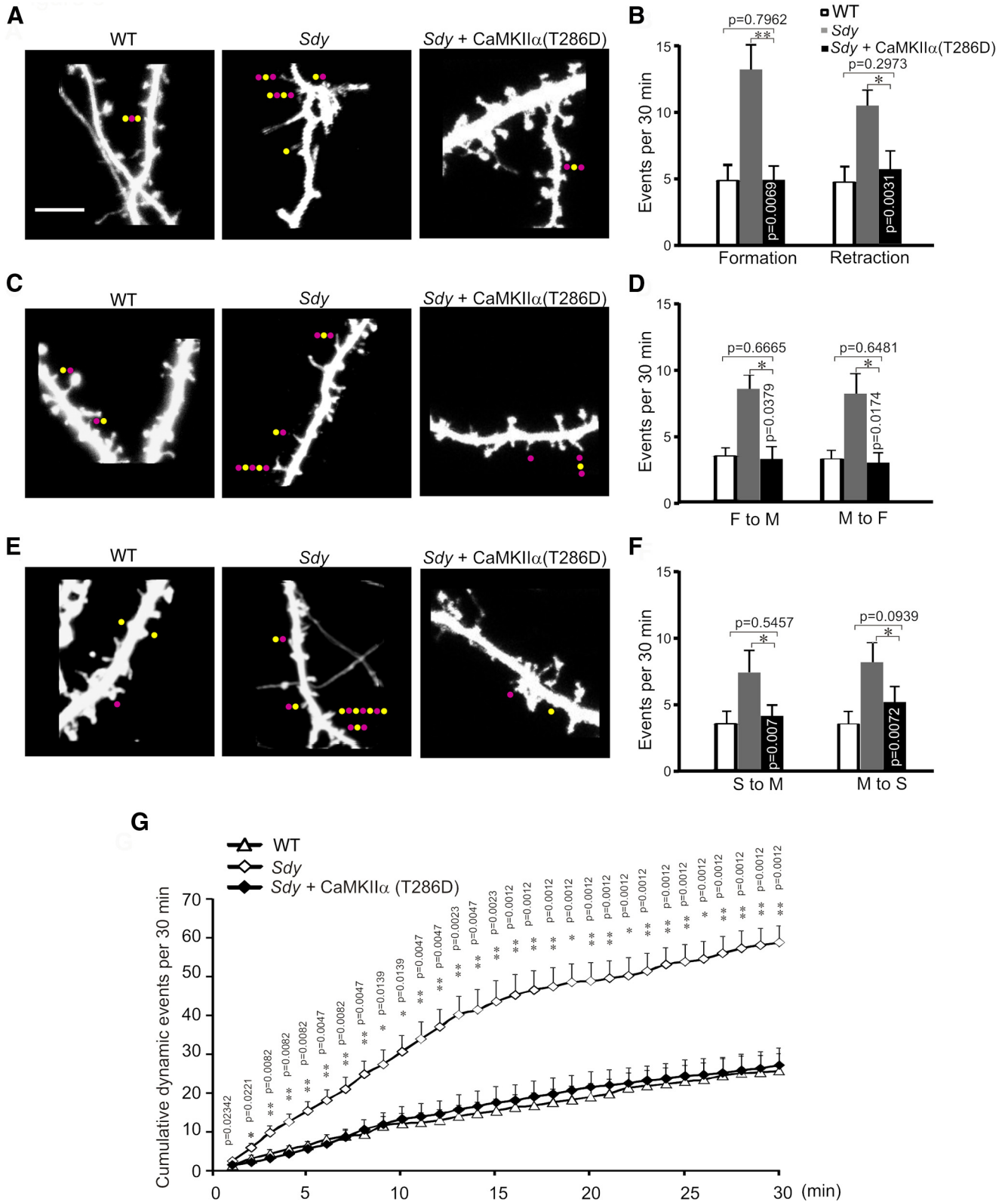
Figure 5. CaMKIIα(T286D) corrects the hyperactivity of dendritic protrusions in *sdy* neurons. Hippocampal neurons from wild-type or *sdy* mice were transfected at 14 DIV with the Venus construct alone or along with the CaMKIIα(T286D) construct, and imaged every minute for 30 min at 17 DIV. *A*, *C*, *E*, Representative images superimposed from those taken at four contiguous time points during the 30 min live-imaging period. Red dots represent protrusion retraction (*A*), the transformation from mushroom/thin spines to filopodia (*C*), the conversion from mushroom/thin to stubby spines (*E*); yellow dots indicate protrusion addition (*A*), the transformation from filopodia to mushroom/thin spines (*C*), the conversion from stubby to mushroom/thin spines (*E*). *B*, *D*, *F*, Quantification of *A*, *C*, *E*. *G*, Cumulative frequency plot for the total number of all types of dynamic events taking place during the 30 min period. Scale bar, 5 μm. Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$.
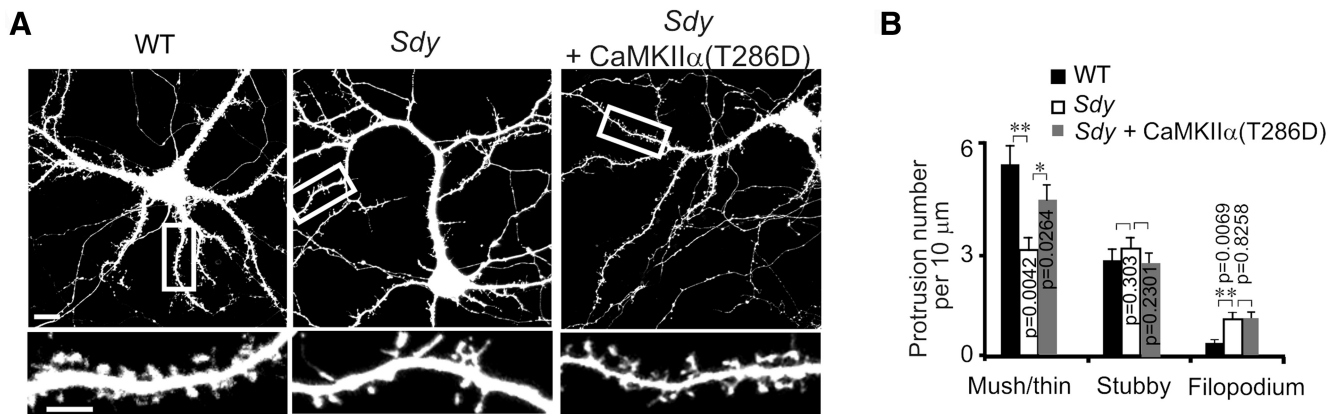
Figure 6. CaMKIIα restores spine density in *sdy* neurons. Cultured hippocampal neurons from *sdy* or wild-type mice were transfected with the Venus construct alone or along with the CaMKIIα(T286D) construct at 14 DIV and imaged at 17 DIV. **A,** Representative images of neurons (top) and dendrites (bottom). **B,** Quantification for **A**; n = 15 neurons for each group. Data are presented as mean ± SEM; *p < 0.05, **p < 0.01.
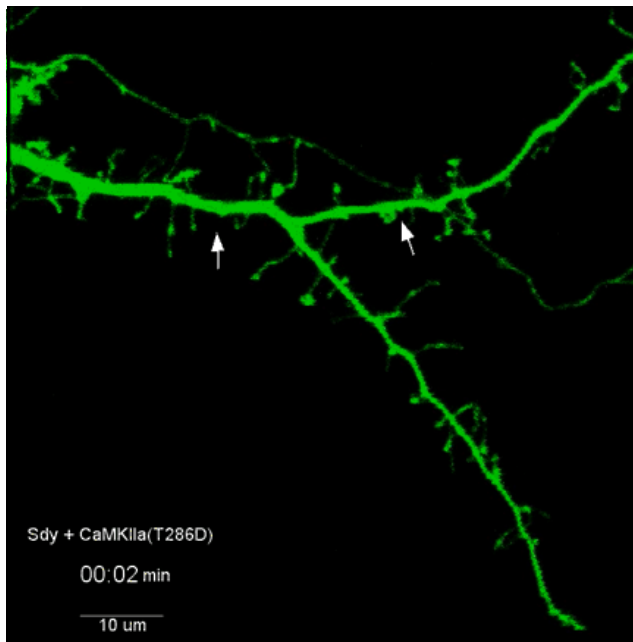
Movie 3. Dynamics of dendrite protrusions in *sdy* neurons. Hippocampal neurons from *sdy* mice were cotransfected with the Venus and the CaMKIIα(T286D) constructs at 14 DIV. This movie shows confocal images of transfected dendrites taken every minute for 30 min at 17 DIV.

level of CaMKIIα (a major isoform of CaMKII) is reduced in the prefrontal cortex (Papaleo et al., 2012), it was unchanged in the hippocampus (Fig. 3 A,B). As CaMKIIα is a synaptic protein, we also analyzed CaMKIIα in the P2 fraction (crude synaptosomal fraction, enriched for presynaptic and postsynaptic proteins), and found that synaptic CaMKIIα was deceased (Fig. 3 A,B), likely due to fewer mushroom/thin spines (which accommodate mature synapses) in *sdy* neurons. In addition, the active form of CaMKIIα (phosphorylated at Thr286) was also reduced in both the whole-cell lysate and the P2 fraction of the *sdy* hippocampus (Fig. 3 A,B). These results indicate that the activity of CaMKIIα is lower in the hippocampus of *sdy* mice.

We next tested whether CaMKIIα affects the dynamics of dendritic protrusions. We cotransfected cultured hippocampal neurons (wild-type, 14 DIV) with the Venus construct and siRNAs against CaMKIIα (See Material and Methods) to knock down CaMKIIα, or a construct expressing constitutively active

CaMKIIα (T286D, CA-CaMKIIα) to elevate CaMKII activity. At 17 DIV, neurons were imaged every 1 min for 30 min. We detected more addition and retraction of dendritic protrusions, conversion of mushroom/thin spines to filopodia, and total dynamic events in CaMKIIα siRNA transfected than in control cells (Fig. 3C–I). Transfection of CA-CaMKIIα also increased the conversion of mushroom/thin spines to filopodia, but had no effect on other types of dynamic events (Fig. 3C–I), possibly due to abundant endogenous CaMKII in synapses (Andersen et al., 2007). Thus, the physiological level of CaMKII activity is required for the stabilization of mushroom/thin spines. Consistent with CaMKIIα's preferential effect on the transformation of mushroom/thin spines, while the densities of filopodia and stubby spines were intact in CA-CaMKIIα or CaMKIIα siRNA-transfected cells, that of mushroom/thin spines was increased in CA-CaMKIIα-transfected, but decreased in CaMKIIα siRNA-transfected neurons (Fig. 4).

Together, these results indicate that CaMKIIα regulates the addition and retraction of dendritic protrusions and the stabilization of mushroom/thin spines.

Decreased CaMKIIα activity in *sdy* mice contributes to the hyperactivity of dendritic protrusions

Recognizing that CaMKIIα regulates the dynamics of dendritic protrusions, and its activity is reduced in *sdy* mice, we next investigated whether lower CaMKIIα activity might be responsible for the excessive dynamics of dendritic protrusions in these mice. To test this possibility, we transfected cultured hippocampal neurons (14 DIV) prepared from *sdy* mice with the CA-CaMKIIα along with the Venus construct, and took time-lapse images at 3 d after transfection. Notably, transfection of CA-CaMKIIα abolished changes to the dynamics of dendritic protrusions and the number of mushroom/thin spines in *sdy* cells (Figs. 5–6; Movies 2, 3). Together, these results indicate that the hyperactivity of dendritic protrusions in *sdy* mice is caused, at least in part, by reduced CaMKIIα activity.

More CaMKIIα binds to Abi1 in *sdy* mice

Having found that CaMKIIα plays an important role in the hyperdynamics of dendritic protrusions in *sdy* mice, we proceeded to investigate why CaMKIIα activity is lower in these mice. Activity-dependent modulation of Abi interactor 1 (Abi1) binds to and inhibits CaMKIIα (Park et al., 2012). Abi1 also forms complexes with dysbindin-1 and the WASP family
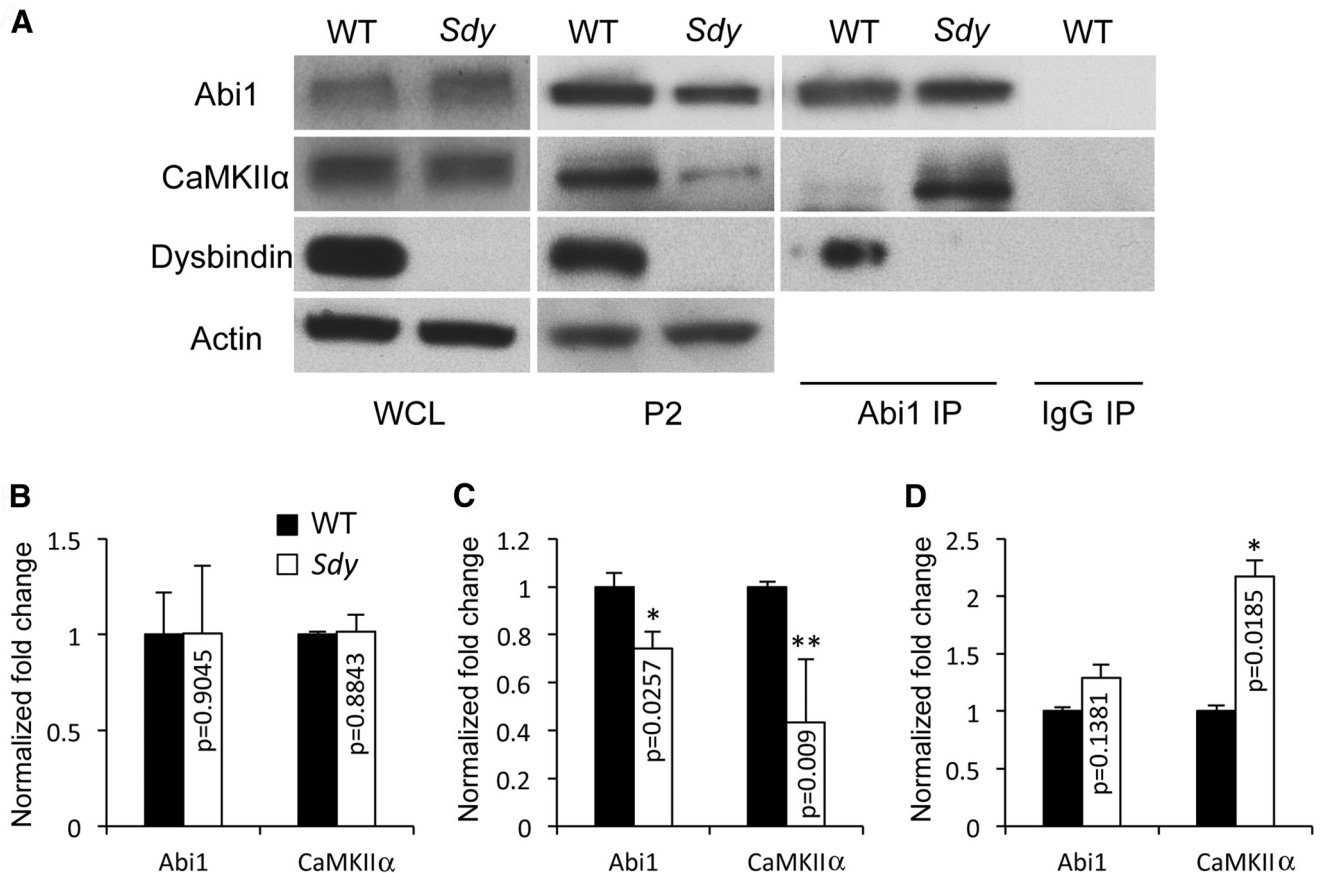
Figure 7. Abi1 forms more complexes with CaMKIIα in *sdy* mice. The hippocampal tissue removed from WT and *sdy* mice (3-week-old) was used for coimmunoprecipitation with an antibody against Abi1. *A*, Representative immunoblots. *B–D*, Quantification of Abi1 and CaMKα in whole-cell lysates (*B*), the P2 fraction (*C*) and the immunoprecipitate (*D*). Data represent the average of three independent experiments using three pairs of wild-type and *sdy* mice, and are presented as mean ± SEM; *p < 0.05, **p < 0.01.

verprolin homology isoform 2 (WAVE2; Ito et al., 2010). In *sdy* mice, the lack of dysbindin can eliminate the Abi1/dysbindin complex, and therefore might result in more Abi1/CaMKIIα complexes and inhibition of CaMKIIα. To test this possibility, we analyzed CaMKIIα associated with Abi1 in *sdy* and wild-type mice using coimmunoprecipitation with an antibody against Abi1. We noticed that although the protein level of Abi1 in the whole-cell lysate was unchanged, it was decreased in the P2 fraction of *sdy* hippocampus (Fig. 7), likely caused by the reduction of mushroom/thin spines. Even the *sdy* hippocampus had less synaptic Abi1, the Abi1 antibody pulled down more CaMKIIα from the P2 fraction of *sdy* than from that of wild-type hippocampi (Fig. 7). These results indicate that the interaction of CaMKIIα and Abi1 is augmented in *sdy* mice. Hence, lower CaMKIIα activity in *sdy* mice is caused, at least in part, by Abi1-mediated inhibition.

Abi1 contributes to the hyperdynamics of dendritic protrusions in *sdy* mice

In *sdy* neurons, CA-CaMKIIα can rescue the number of mushroom/thin spines, but has no effect on filopodium overgrowth (Figs. 5, 6). Hence, CaMKII-independent mechanisms may also contribute to *sdy* mice's protrusion phenotype. Dysbindin binds to and facilitates the interaction of Abi1 and WAVE2 (a regulator of actin reorganization; Ito et al., 2010; Rotty et al., 2013). Knockdown of dysbindin causes a reduction in the formation of the Abi1/WAVE2 complex and presumably affects actin dynamics, a process important for the morphogenesis of dendritic protru-

sions (Li and Sheng, 2003; Ito et al., 2010). In addition, we found that synaptic expression of Abi1 is reduced (Fig. 7) and it has been reported that Abi1 overexpression can restore the density and morphology of dendritic protrusions in dysbindin knockdown neurons (Ito et al., 2010). We therefore tested whether Abi1 also contributes to the changes in protrusion dynamics in *sdy* mice.

We transfected primary hippocampal neurons prepared from *sdy* mice with a construct expressing Abi1, and took time-lapse images of transfected dendrites as described above. Abnormal protrusion dynamics in *sdy* neurons including formation, retraction, and conversion between different types of protrusions were all normalized by transfection of the Abi1 construct (Fig. 8). The changes to the densities of mushroom/thin spines and filopodia in *sdy* neurons were also obliterated by Abi1 overexpression (Fig. 9). As CaMKIIα does not, whereas Abi1 can, rescue filopodium density in *sdy* neurons, Abi1 can regulate dendritic protrusions through CaMKIIα-independent mechanisms, such as actin reorganization.

Discussion

In this study, we used time-lapse imaging to investigate the role of the schizophrenia risk gene dysbindin in the morphogenesis of dendritic spines. Our results show that dysbindin regulates the dynamics of dendritic protrusions. In *sdy* mice, formation, retraction, and transformation of dendritic protrusions are more active. We also found that CaMKIIα is required for the stabilization of dendritic protrusions, and that
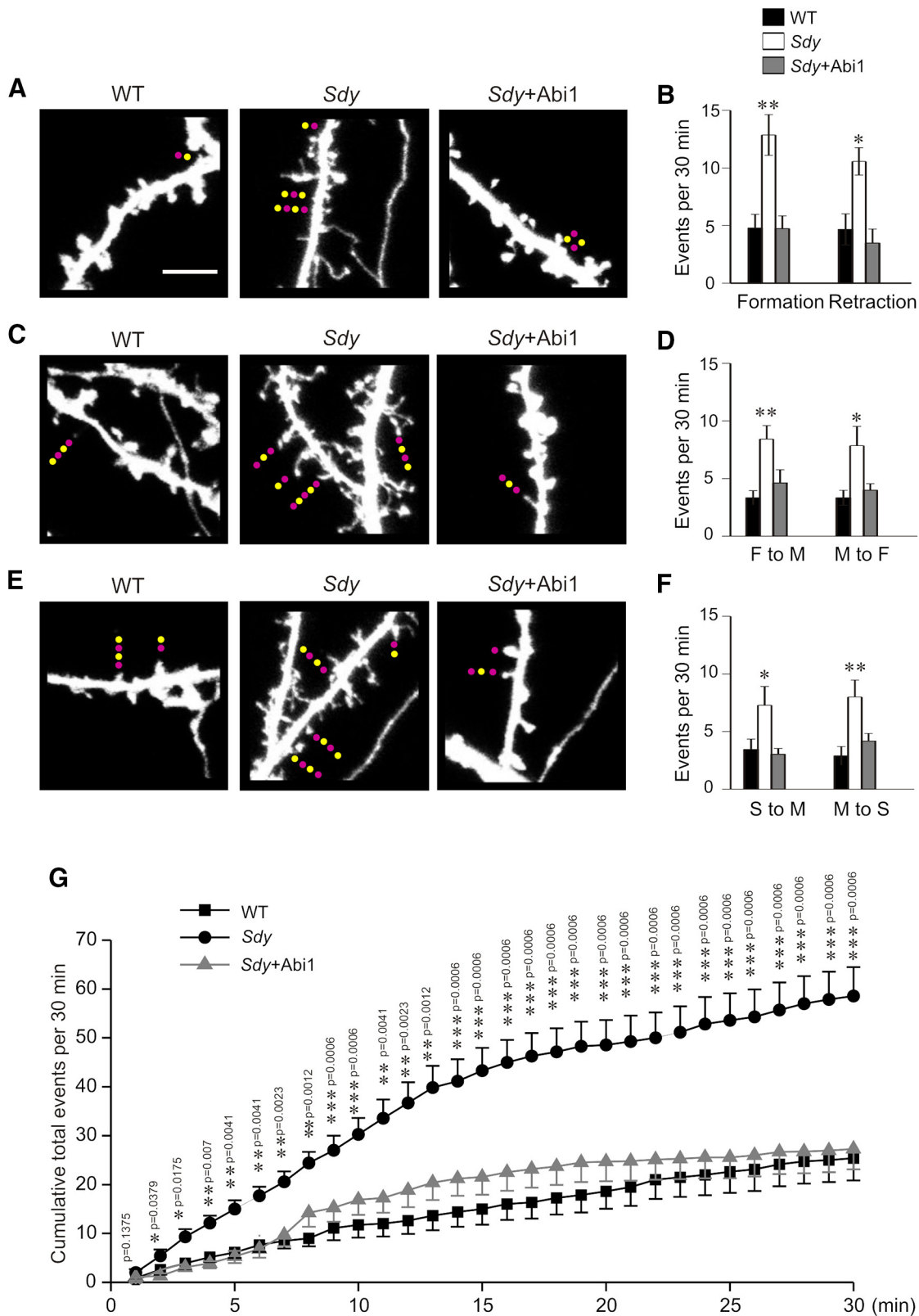
Figure 8. Abi1 eliminates protrusion hyperdynamics of *sdy* neurons. Hippocampal neurons from wild-type and *sdy* mice were transfected at 14 DIV with the Venus construct alone or along with the Abi1 construct, and imaged every minute for 30 min at 17 DIV. ***A***, ***C***, ***E***, Representative images superimposed from those taken at four contiguous time points during the 30 min live-imaging period. Red dots represent protrusion retraction (***A***), the transformation from mushroom/thin spines to filopodia (***C***), the conversion from mushroom/thin to stubby spines (***E***); yellow dots indicate protrusion addition (***A***), the transformation from filopodia to mushroom/thin spines (***C***), the conversion from stubby to mushroom/thin spines (***E***). ***B***, ***D***, ***F***, Quantification of ***A***, ***C***, ***E***. ***G***, Cumulative frequency plot for the total number of all types of dynamic events taking place during the 30 min period. Scale bar, 5 μm. Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
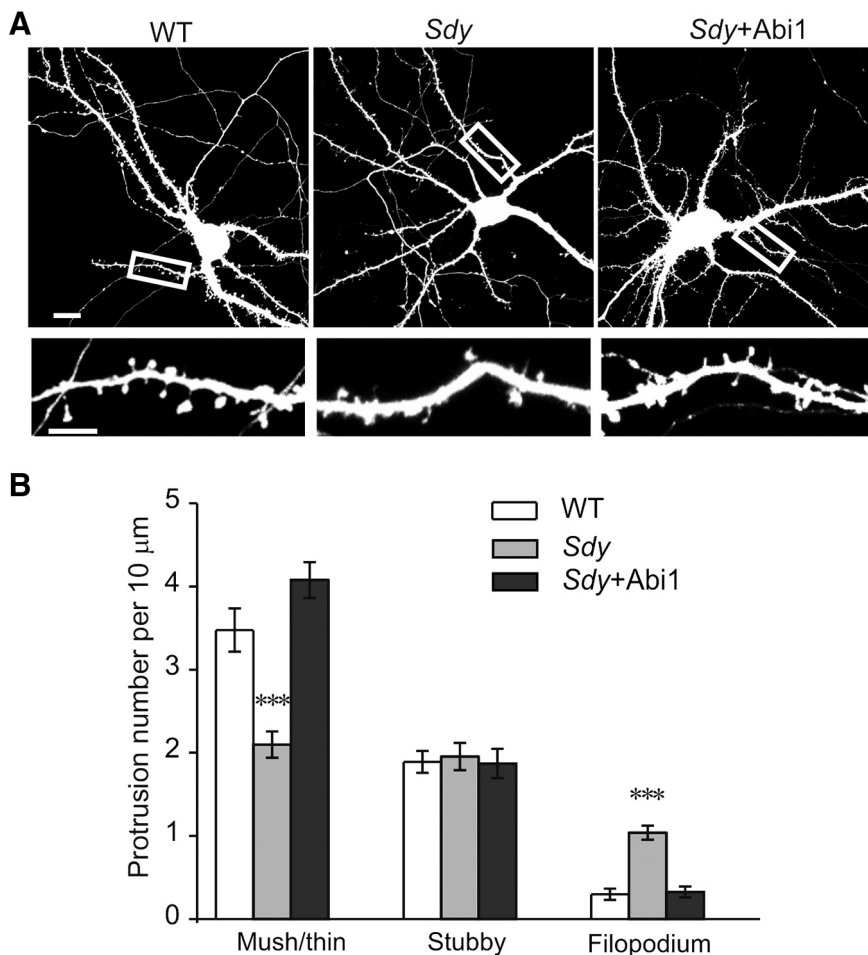
A

WT   Sdy   Sdy+Abi1

B

Figure 9. Abi1 restores the densities of mushroom/thin spines and filopodia in *sdy* neurons. Cultured hippocampal neurons from *sdy* or wild-type mice were transfected with the Venus construct alone or along with the Abi1 construct at 14 DIV and imaged at 17 DIV. **A**, Representative images of neurons (top) and dendrites (bottom). **B**, Quantification for **A**; $n = 15$ neurons for each group. Data are presented as mean $\pm$ SEM; ***$p < 0.001$.

protrusion dynamics, indicating that CaMKII activity is required to stabilize dendritic protrusions. This finding is consistent with a previous one from the barrel cortex of mice that CaMKII activity is required for whisker trimming-induced stabilization of new spines (Wilbrecht et al., 2010). Hence, CaMKII is essential for spine stabilization in both development and experience-dependent plasticity of spines. Last and perhaps more importantly, constitutively active CaMKIIα restores protrusion stability in *sdy* mice.

We found that, likely due to CaMKII's stabilizing effect on spines, expression of constitutively active CaMKIIα increases the number of mushroom/thin spines. By contrast, the number of stubby spines is not changed by constitutively active CaMKIIα. The different responses of stubby and mushroom/thin spines to CaMKII overactivation could be due to the tendency of stabilized stubby spines to turn into mushroom/thin spines (which predominate in mature neurons), or perhaps to a high sensitivity CaMKII activity that would allow even small endogenous amounts of CaMKII to have already elicited stubby spines' maximal response.

It should be noted that knockdown of CaMKIIα only increases the conversion of mushroom/thin spines to filopodia, whereas transfection of CA-CaMKIIα corrects all abnormal spine dynamics in *sdy* neurons. These findings suggest that multiple factors in addition to CaMKIIα (some of which have overlapping function with CaMKIIα) contribute to the protrusion phenotype of *sdy* mice. Our data show that one such factor is Abi1. By binding to CaMKII, Abi1 causes a decrease in CaMKIIα activity and consequent changes to the dynamics and number of dendritic protrusions in *sdy* mice. In addition, Abi1 also modulates protrusions through CaMKIIα-independent pathways, as Abi1 overexpression in *sdy* neurons abolishes filopodium overgrowth, a phenotype unchanged by expression of constitutively active CaMKIIα. The multifaceted mechanisms used by Abi1 to regulate protrusions is conferred by its diverse binding partners including CaMKIIα (stabilizing dendritic protrusions) and WAVE2 (which activates actin-related proteins 2 and 3 to regulate actin dynamics; Rotty et al., 2013).

CaMKII is essential for cognitive functions. Mice with reduced CaMKIIα expression exhibit behavioral abnormalities including deficits in working memory which is an endophenotype of schizophrenia (Yamasaki et al., 2008). Our findings of CaMKII's role in abnormal protrusion dynamics of *sdy* neurons suggest that the behavioral impairment of these mice may in part stem from the "miswiring" of neural circuits resulting from the destabilization of dendritic protrusions during brain development.

In summary, by examining the dynamic morphogenesis of dendritic protrusions using time-lapse imaging, we demonstrate that dysbindin and CaMKII are key regulators involved in the

reduced CaMKIIα activity in *sdy* mice contributes to the hyperactivity of protrusions.

Because genetic variants of the *dysbindin* gene are associated with risks of schizophrenia, there is a keen interest in the biology of dysbindin. Using *sdy* mice and dysbindin siRNAs, several groups including us show that dysbindin is required for spine development. The specific stage of spine development that is regulated by dysbindin, however, has yet to be elucidated. Our findings of excessive addition, retraction, and transformation of dendritic protrusions in *sdy* neurons suggest that dendritic protrusions and synaptic connections formed on them are highly unstable in these cells. Destabilization of protrusions can hamper their maturation. Indeed, consistent with previous reports (Jia et al., 2013), we found that the number of mature spines (mushroom and thin spines) is reduced in *sdy* neurons. It is possible that the dysbindin gene contributes to the synaptopathology of schizophrenia by destabilizing dendritic spines during neuronal development.

How does dysbindin regulate spine dynamics? A few lines of evidence from our study suggest that CaMKII is a mediator for the regulation of spine dynamics by dysbindin. First, phosphorylated CaMKIIα (the active form of CaMKIIα) is decreased in the hippocampus of *sdy* mice due to excessive formation of the Abi1/CaMKIIα complexes. Second, the CaMKIIα siRNA enhances

stabilization of dendritic protrusions. Our findings also suggest that reduced dysbindin expression might contribute to the synaptopathology of schizophrenia by destabilizing dendritic protrusions and synaptic connections on them.

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