DTNBP1, a schizophrenia susceptibility gene, affects kinetics of transmitter release

Xiao-Wei Chen,1,2 Ya-Qin Feng,3 Chan-Juan Hao,3 Xiao-Li Guo,3 Xin He,3 Zhi-Yong Zhou,3 Ning Guo,1,2 Hong-Ping Huang,1,2 Wei Xiong,1,2 Hui Zheng,1,2 Pan-Li Zuo,1,2 Claire Xi Zhang,1,2 Wei Li,3 and Zhuan Zhou1,2

1Institute of Molecular Medicine and 2State Key Laboratory of Biomembrane Engineering, Peking University, Beijing 100871, China
3Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Schizophrenia is one of the most debilitating neuropsychiatric disorders, affecting 0.5–1.0% of the population worldwide. Its pathology, attributed to defects in synaptic transmission, remains elusive. The dystrobrevin-binding protein 1 (DTNBP1) gene, which encodes a coiled-coil protein, dysbindin, is a major susceptibility gene for schizophrenia. Our previous results have demonstrated that the sandy (sdy) mouse harbors a spontaneously occurring deletion in the DTNBP1 gene and expresses no dysbindin protein (Li, W., Q. Zhang, N. Oiso, E.K. Novak, R. Gautam, E.P. O’Brien, C.L. Tinsley, D.J. Blake, R.A. Spritz, N.G. Copeland, et al. 2003. Nat. Genet. 35:84–89). Here, using amperometry, whole-cell patch clamping, and electron microscopy techniques, we discovered specific defects in neurosecretion and vesicular morphology in neuroendocrine cells and hippocampal synapses at the single vesicle level in sdy mice. These defects include larger vesicle size, slower quantal vesicle release, lower release probability, and smaller total population of the readily releasable vesicle pool. These findings suggest that dysbindin functions to regulate exocytosis and vesicle biogenesis in endocrine cells and neurons. Our work also suggests a possible mechanism in the pathogenesis of schizophrenia at the synaptic level.

Introduction

Schizophrenia is one of the most devastating neuropsychiatric diseases. Pharmacological and neuropathological evidence suggests that dysfunction of dopaminergic, glutamatergic, or GABAAergic transmission underlies its symptomatology (Lewis et al., 2005; Coyle, 2006; Ross et al., 2006; Snyder, 2006). Genetic epidemiological studies show that schizophrenia has a heritability of ~80% (Cardno and Gottesman, 2000), and several putative schizophrenia-susceptibility genes have been identified (Norton et al., 2006; Ross et al., 2006). Among these, the dystrobrevin-binding protein 1 (DTNBP1) or dysbindin gene, located on chromosome 6p, is a promising candidate from schizophrenia linkage and association studies (Straub et al., 1995; Wang et al., 1995; O’Donovan et al., 2003; Schwab et al., 2003; Norton et al., 2006; Morris et al., 2008).

Compelling evidence indicates that dysbindin influences neurotransmission and so contributes to the cognitive dysfunctions in schizophrenia. In most patients with schizophrenia, the expression level of dysbindin is reduced in the hippocampus and prefrontal cortex, two regions that have long been associated with psychotic symptoms (Talbot et al., 2004; Weickert et al., 2004; Weickert et al., 2008). However, no protein coding sequence mutations of the dysbindin gene have been found (Ross et al., 2006). Knockdown of dysbindin in cultured cells affects SNAP25 expression and the extracellular glutamate or dopamine levels (Numakawa et al., 2004; Kumamoto et al., 2006). More recently, it has been shown that the sandy (sdy) mouse, which harbors a large deletion within the dysbindin gene and has no dysbindin protein expression (Li et al., 2003), has reduced dopamine levels in the cortex and hippocampus (Murotani et al., 2007). Furthermore, dysbindin is located on the synaptic vesicles, postsynaptic densities (PSDs), and microtubules of apparent glutamatergic neurons in the hippocampus. It binds to snapin (Starcevic and Dell’Angelica, 2004; Talbot et al., 2006), a SNARE-associated protein implicated in neurotransmission.
Results

Lack of dysbindin changes kinetics of quantal vesicle release in chromaffin cells

Amperometry provides high temporal and spatial resolution in directly detecting vesicular release from various cell types (Wightman et al., 1991; Augustine and Neher, 1992; Chow et al., 1992; Artalejo et al., 1994; Albillos et al., 1997; Chen et al., 2005). Amperometric measurements have another advantage, as they detect exclusively presynaptic release without contamination from the postsynapse. Therefore, taking advantage of amperometric recordings in the adrenal slice, a physiological and widely used model for studying stimulus-secretion coupling (Moser and Neher, 1997; Barbara et al., 1998), we first determined whether dysbindin regulates the characteristics of single vesicle fusion events. Local application of high-K⁺ solution elicited
amperometric spikes in a wild-type (WT) chromaffin cell (Fig. 1A). Three parameters of amperometric spikes were analyzed: half-height duration (HHD) and rise time (RT), which reflect the fusion kinetics; and quantal size (Q), which reflects the vesicle content released in each spike (Fig. 1C). The shape of spikes changed markedly, with a longer HHD (WT, 13 ± 1 ms; sdy, 20 ± 2 ms) and a larger Q (WT, 0.52 ± 0.05 pC; sdy, 0.68 ± 0.06 pC) in sdy cells (Fig. 1, B and C). However, no noticeable difference was detected in RT (WT, 3.1 ± 0.4 ms; sdy, 3.2 ± 0.3 ms). These data suggest that dysbindin plays a role in the regulation of single vesicle release kinetics and Q in chromaffin cells.

To test whether dysbindin also regulates the total amount and probability of release, whole-cell patch-clamped cells were stimulated by 2-s depolarization pulses, and amperometric signals and membrane currents were simultaneously monitored. Compared with WT cells, the evoked total amperometric spike charge was reduced by ~30% in sdy cells (WT, 49 ± 8 pC; sdy, 32 ± 4 pC; Fig. 1, D and E). Similarly, sdy cells displayed a modest reduction in the number of amperometric spikes (WT, 20 ± 3 events per cell; sdy, 14 ± 1 events per cell), which suggests a decreased vesicle release probability caused by dysbindin deficiency (Fig. 1, D and E). In contrast, voltage-gated Na⁺ and Ca²⁺ currents were unaffected (Fig. 1, D and E), demonstrating that the observed secretory defects in the absence of dysbindin were not caused by changes in Ca²⁺ influx. Collectively, these amperometric data suggest that dysbindin regulates large dense-core vesicle (LDCV) secretion in terms of total amount of secretion, vesicular release probability, and the kinetics and size of single fusion events.

**Figure 2. Microdialysis of purified full-length dysbindin rescues the slow kinetics and reduced Q but not the probability and total amount of release in sdy mice.** (A) Time course of the effects of intracellular dysbindin (black line) or heat-denatured dysbindin (gray line) on the kinetics of amperometric spikes and Q in sdy chromaffin cells. After establishing whole-cell mode, amperometric spikes were elicited every 5 min by a 2-s depolarization from −70 to 0 mV. The first points (−) represent data before dysbindin dialysis in sdy cells. The second, third, and fourth data points correspond to 1, 5, and 10 min after establishing whole-cell recording with 0.2 μg/μl full-length dysbindin or the denatured protein in the patch pipette. Note the appearance of fast kinetics of amperometric spikes at 5 min after dysbindin dialysis and the lack of difference between 5 and 10 min. In WT control, diamond data points indicate the mean values of amperometric spikes obtained at 5 and 10 min without dysbindin treatment. n = 201 spikes (−); 13 cells, 2 sdy mice; 116 spikes (1 min); 11 cells, 2 sdy mice; 94 spikes (5 min), 81 spikes (10 min), and 193 spikes (14 cells, 2 WT mice). (B) Averaged amperometric spikes obtained at 5 and 10 min after establishing whole-cell recording in untreated WT (gray) or sdy cells without (black) or with dysbindin addition (dotted line). Each line was averaged from 100 amperometric spikes, respectively. (C) In WT cells, histograms show the quantitative analyses of single spike properties (HHD, RT, and Q) without (gray) or with (striped) 5 and 10 min of intracellular dialysis of dysbindin. Data were obtained from the same adrenal slices from two WT littermates. n = 112 spikes (9 cells, WT) and 111 spikes (11 cells, WT + dysbindin). (D) Histograms show the amount of catecholamine secretion ([Iamp]dt; left), the number of amperometric events per cell (middle), and calcium currents Ica (right) in sdy cells without (black); 13 cells, 2 sdy mice) or with 5 and 10 min dysbindin dialysis (striped; 11 cells, 2 sdy mice). Error bars indicate the mean ± SEM.

Intracellular dialysis of purified full-length dysbindin rescues the sdy phenotype of LDCV secretion

To confirm that the aforementioned phenotype in sdy mice was directly caused by the lack of dysbindin, we intracellularly applied purified full-length dysbindin protein (352 amino acids, RefSeq available from GenBank/EMBL/DBJ under accession no. NM_025772; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200711021/DC1) into chromaffin cells by patch pipette. Similar to previous approaches in which dialysis of glutamate (Wu et al., 2007) or microinjection of peptide (Ilardi et al., 1999; Zhang and Zhou, 2002; Chen et al., 2005; Krapivinsky et al., 2006) for a certain amount of minutes was sufficient to regulate exocytosis in neurons or chromaffin cells, we recorded vesicle release events after dysbindin application over a period of minutes. After stable whole-cell recording was established, amperometric responses were evoked by a 2-s depolarization every 5 min for ~30 min. The temporal effect of dysbindin dialysis on the characteristics of single amperometric spikes is shown in Fig. 2A. Perfusion of 0.2 μg/μl dysbindin for 5 min significantly decreased the HHD and Q in sdy cells. No further
Morphological changes in LDCVs of sdy chromaffin cells

Defects in LDCV secretion might result from changes in number, size, or spatial distribution of chromaffin vesicles. To differentiate among these possibilities, we performed electron microscopy on adrenals from WT and sdy mice. The gross morphology of sdy chromaffin cells was similar to that of the WT (Fig. 3A). The size distribution of LDCVs in sdy mice showed an 10% increase in vesicle diameter (Fig. 3B). Furthermore, counting the total number of vesicles per square micrometer revealed that the vesicle density in sdy cells was markedly lower (by 25%) than that of WT cells (Fig. 3C). However, neither the number of docked vesicles, i.e., those located within 100 nm of the plasma membrane (Fig. 3A, insets), nor the overall spatial distribution of the vesicles differed between WT and sdy cells (Fig. 3D), which suggests that dysbindin is not required for effective docking in chromaffin cells. These defects are consistent with our electrochemical data, namely that increases in vesicle size or volume may be reflected in increases in the amount of transmitter released per vesicle (Q; provided that vesicle luminal concentration remains the same; Bruns et al., 2000; Sulzer and Edwards, 2000; Karunanithi et al., 2002), and the reduced vesicle density may contribute to the decreased vesicular release probability.

Kinetics of synaptic transmission are changed in sdy hippocampus

Because the machinery for regulating exocytosis in chromaffin cells and neurons shares many features, including Ca²⁺ dependence...
and the kinetics of exocytosis (Morgan and Burgoyne, 1997), we investigated whether the lack of dysbindin alters glutamatergic transmission in ventral hippocampal CA1, a brain structure where abnormalities have been reported in schizophrenia (Weinberger, 1999). Here, the pyramidal neurons receive major excitatory inputs from Schaffer and commissural collaterals of CA3 efferents, and dysbindin expression is high (Talbot et al., 2004, 2006). We compared miniature excitatory postsynaptic currents (mEPSCs), which occur spontaneously and normally correspond to single vesicle fusion events, in ventral hippocampal CA1 pyramidal neurons of WT and sdy mice (Fig. 4 A). The amplitude of mEPSCs was not significantly different (WT, 7.4 ± 0.2 pA; sdy cells, 8.1 ± 0.6 pA; Fig. 4 B), which generally indicates that the responsiveness and number of postsynaptic receptors are not altered in the absence of dysbindin (Nicoll and Malenka, 1999). Consistent with this, the AMPA/NMDA ratio did not differ between the two genotypes (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200711021/DC1). In contrast, the frequency of mEPSCs, an indicator of release probability from presynaptic terminals, was reduced from 11 ± 1 events per min in the WT to 6 ± 1 events per min in sdy, as clearly shown in the cumulative frequency distribution (Fig. 4 C).

Analysis of the kinetics of mEPSCs further demonstrated dramatic increases in HHD, RT, decay time constant, and charge in dysbindin-deficient cells (Fig. 4, D and E). These changes depicted slower single vesicle release kinetics but larger quanta in the absence of dysbindin. Collectively, these results suggest that dysbindin modulates the kinetics, amount, and probability of single presynaptic vesicle release in hippocampal CA1 glutamatergic synapses, just as in chromaffin cells. However, unlike in chromaffin cells, we found that the RT was affected in sdy hippocampal neurons. It is possible that this change in RT of mEPSCs is a combined pre- and postsynaptic phenomenon, whereas the quantal analysis by amperometry reflects the pure "presynaptic" release signal without contamination by postsynaptic mechanisms. It is also possible that the function of dysbindin in vesicle exocytosis may be slightly different between neuroendocrine cells and neuronal synapses.

Because we discovered changes in spontaneous single vesicle release, it is plausible that action potential–dependent transmitter release may also be affected. We subsequently examined the evoked EPSCs in hippocampal CA1 pyramidal neurons
by stimulating Schaffer collateral axons. As shown in Fig. 4 (F and G), the absence of dysbindin caused a reduction in EPSC peak amplitude (WT, 264 ± 33 pA; sdy, 189 ± 15 pA) without affecting the charge transfer (WT, 24 ± 8 nC; sdy, 21 ± 5 nC), a measure of the total amount of glutamate release. The RT of EPSCs (WT, 17 ± 3 ms; sdy, 24 ± 3 ms) was slightly higher in sdy neurons, but the change was statistically insignificant. However, a significant increase in the decay time constant (WT, 104 ± 19 ms; sdy, 190 ± 30 ms) was found in the sdy neurons. This is consistent with the mEPSC analysis (Fig. 4, A–E), where the reduced release probability and larger quantal content compensated each other, causing the total amount of release to be unaltered. Therefore, these results indicate that the absence of dysbindin also impairs the kinetics of evoked glutamate release in hippocampal CA1.

Morphological changes in asymmetrical synapses of sdy hippocampus

Abnormal hippocampal cytoarchitecture, such as synaptic abnormalities in glutamatergic terminals, has been reported in patients with schizophrenia (Harrison and Eastwood, 2001). Because we here found several defects in glutamate vesicle release in hippocampal CA1, combined with the fact that dysbindin reduction occurs in the glutamatergic terminals of the hippocampus in schizophrenia (Talbot et al., 2004), we set out to examine the excitatory asymmetrical synapses on CA1 dendritic spines in sdy mice by electron microscopy. The overall appearance of presynaptic terminals and spines in sdy mice appeared normal and no gross defect was detected (Fig. 5 A, top). Quantitative analysis revealed that the absence of dysbindin resulted in a shift of the vesicle distribution to ~10% larger size, averaging 43.8 ± 0.1 nm (WT) and 46.9 ± 0.1 nm (sdy; Fig. 5 B).

In an attempt to understand whether dysbindin functions in different vesicle pools, we counted the numbers of vesicles in a docked state, those immediately (~50 nm) next to the active zone membrane (Fig. 5 A, bottom; Pozzo-Miller et al., 1999), and those of the reserve pool, located farther than ~50 nm from the active zone. We did not find any abnormality in the docked vesicles in sdy mice (Fig. 5 C), which suggests that dysbindin is not involved in the docking process in the hippocampus. In contrast, the density of reserve pool vesicles was reduced by ~22% in sdy animals (Fig. 5 C), which is consistent with the defect seen in adrenal morphology (Fig. 3). Interestingly, we also discovered decreased width of the synaptic cleft in sdy (Fig. 5 C). This probably reflects a compensatory change following the presynaptic defects within the hippocampus. In addition, sdy synapses exhibited increased thickness of PSDs (Fig. 5 C). Collectively, these results demonstrate that a lack of dysbindin leads to larger but fewer glutamatergic vesicles, narrower synaptic clefts, and thicker PSDs within CA1 asymmetrical synapses, in agreement with our electrophysiological observations of slow dynamics and reduced probability of glutamate release (Fig. 4).

Smaller RRP size in sdy chromaffin cells and hippocampus

Abnormal vesicle structure and slower vesicle release kinetics in sdy cells might result from defective vesicle endocytosis and recycling (Deak et al., 2004). Therefore, cell membrane capacitance (Cm) measurements, which can be used for endocytosis
recording (Smith and Neher, 1997; Zhang et al., 2004), were made in chromaffin cells to determine whether endocytosis was affected in the absence of dysbindin. Compared with WT cells, a 200-ms depolarizing pulse from −70 to +10 mV induced an ∼30% decrease in the ΔCm response in sdy cells (WT, 485 ± 109 fF; sdy, 337 ± 25 fF; Fig. 6 A), reflecting the exocytosis of LDCVs. This result is consistent with the total amount of secretion obtained from amperometric recordings, showing a ∼30% reduction in amperometric spike charge in sdy cells (Fig. 1, D and E). After reaching a peak, the Cm trace declined, representing endocytosis. We found that the kinetics of endocytosis in sdy cells were not significantly different from those in WT cells (decay time constant: WT, 173 ± 69 s; sdy, 186 ± 85 s), which suggests that the endocytosis of LDCVs is normal in dysbindin-deficient chromaffin cells.

The RRP is generally considered to be the fusion-competent population of vesicles. The size of the RRP in chromaffin cells was estimated by using a dual-pulse protocol (Gillis et al., 1996; Smith et al., 1998), which is designed to elicit and measure secretory depression through two identical Ca2+ current injections given in rapid succession (Fig. 6 B). The RRP size, Bmax, is derived from the equation: Bmax = S/(1 − R2). S represents the sum of the capacitance responses to the first (∆Cm1) and the second (∆Cm2) pulse, and R is defined as the ratio ∆Cm2/∆Cm1. A value of R < 1 reflects secretory depression, presumably owing to depletion of the RRP. Analyses were limited to R < 0.7 for an accurate estimate (Gillis et al., 1996; Smith et al., 1998). Typical responses obtained from WT and sdy cells are shown in Fig. 6 B. Analysis showed that the RRP size in sdy cells was only ∼50% of that in WT cells (Fig. 6 C). To investigate the time course of RRP recovery from depletion, a second dual pulse was applied at different time intervals to probe the pool size. The refilling kinetics can be estimated by a monoexponential fit of the interpulse interval versus the normalized RRP size. As shown in Fig. 6 D, the time courses of recovery were similar in WT and sdy cells (time constant of recovery: WT, 3.3 s; sdy, 2.8 s), indicating that dysbindin deficiency does not affect the refilling kinetics of the RRP.

In addition, trains of high-frequency stimuli were applied to estimate the RRP size and the rate of replenishment of the RRP in hippocampal CA1 pyramidal neurons (Schneggenburger et al., 1999). A train of 40 pulses delivered at 20 Hz induced facilitation of EPSCs between the first and fourth pulses, followed by depression (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200711021/DC1). To estimate the RRP size, we plotted the cumulative EPSC charge during the train and extrapolated back to time = 0 to determine the RRP (Schneggenburger et al., 1999). As shown in Fig. S3 A, sdy neurons had a smaller RRP than WT neurons (WT, 105 ± 24 pC; sdy, 73 ± 15 pC). Because the size of the RRP correlates with release probability in hippocampal synapses (Dobrunz and Stevens, 1997; Dobrunz, 2002), this result from chromaffin cells is in agreement with the observation (shown in Fig. 6) that release probability is reduced in sdy synapses. However, the kinetics of replenishment of the RRP were not significantly different in CA1 neurons of either genotype (Fig. S3 B). Collectively, these findings indicate that dysbindin deficiency results in a smaller RRP of vesicles without affecting the refilling kinetics of the RRP in chromaffin cells and hippocampal neurons.
Normal expression levels of key components of synaptic vesicle release machinery in sdy adrenal gland and hippocampus

To understand whether other related proteins are affected in sdy mice, we next investigated the expression levels of major proteins involved in synaptic vesicle release. Western blot analyses of hippocampus and adrenal gland homogenates confirmed that the expression of dysbindin was abolished in the sdy mouse (Fig. 7). Dysbindin in the WT hippocampus existed in two isoforms (50 and 38 kD), and the 38-kD isoform was predominant. In adrenal glands, the 50-kD isoform was the only one, as in kidney (Li et al., 2003). However, we did not find any changes in semiquantitative assays (P > 0.05) in the steady-state levels of a panel of known synaptic proteins, including t-SNAREs (SNAP25 and syntaxin-1), intrinsic membrane proteins of synaptic vesicles (synaptotagmin/VAMP2, synaptotagmin-1, and VGLUT-1), and vesicle-associated proteins (dynamin I, complexin1/2, and munc18-1). Therefore, lack of dysbindin does not cause evident changes in the expression of these proteins. In addition, we did confirm that dysbindin binds to snapin (unpublished data), which is known to bind SNAP25 (Buxton et al., 2003; Ilardi et al., 1999).

Discussion

Recent studies have led to the suggestion that dysbindin, a putative susceptibility factor for schizophrenia, may play a role in neurotransmission (Numakawa et al., 2004; Kumamoto et al., 2006; Talbot et al., 2006), but very little is known about the phenotypes at the single vesicle level. In this study, using functional and morphological approaches in a spontaneously occurring dysbindin-deficient animal (sdy), we identified several phenotypes of the dysbindin in neurosecretion at the single vesicle and synaptic levels as follows: (1) the single vesicle catecholamine secretion was slower in kinetics, larger in Q, and lower in release probability/total secretion in sdy chromaffin cells; (2) exogenous dialysis of purified dysbindin partially rescued the abnormalities of secretion; (3) the duration and Q of single synaptic vesicle release were increased in sdy hippocampal CA1 nerve terminals; (4) electron microscopy analyses illustrated larger vesicle size and lower vesicle density in both hippocampal nerve terminals and chromaffin cells; (5) sdy chromaffin cells had a smaller RRP size but normal RRP refilling kinetics; (6) and finally, immunoblot analyses showed that the expression of a panel of known regulatory components of the vesicle exocytosis was not changed in sdy mice.

Our current hypothesis is that dysbindin may play multiple roles in regulating the synaptic vesicle cycle. In general, vesicles go through three steps during exocytosis: docking, priming, and fusion. After exocytosis, vesicle membrane components rapidly and selectively undergo endocytosis and recycling processes that make them competent for the next round (Hannah et al., 1999; Sudhof, 2004). Therefore, changes in the kinetics of single vesicle release in dysbindin-deficient cells suggest that this may affect the fusion process (i.e., fusion pore dynamics), as the slowing of quantal release kinetics (increased HHD and decay time) is consistent with a function of dysbindin in modulating fusion pore closure to allow kiss-and-run exocytosis (Zhou et al., 1996; Burgoyn and Barclay, 2002; Chen et al., 2005). This change in fusion pore dynamics may also contribute to the reduced release probability (Burgoyn and Barclay, 2002). Dysbindin may affect vesicle genesis because the vesicle size and quantal release was larger in sdy cells (Figs. 1–5). Furthermore, dysbindin deficiency reduced the size of the RRP without changing endocytosis or vesicle pool refilling. This result, together with the normal morphology of docked vesicles in sdy mice, also suggests a postdocking function of dysbindin, probably in the priming process (i.e., the stability of release-ready vesicles).

It is conceivable that these functions of dysbindin in the priming and fusion steps may be mediated by its interaction with the SNARE proteins and/or other synaptic vesicle proteins involved in exocytosis (Burgoyn and Barclay, 2002; Bonanomi et al., 2006). In our experiments, we did not find any changes in the steady-state levels of these exocytotic regulators, including SNARE proteins (SNAP25, syntaxin-1, and VAMP2), synaptotagmin-1, VGLUT1, munc18-1, and complexin1/2 (Fig. 7). However, we did confirm that snapin is a binding partner of dysbindin (unpublished data; Talbot et al., 2006). Snapin is identified as a ubiquitously expressed SNAP25-binding protein (Ilardi et al., 1999; Buxton et al., 2003). It has been proposed that snapin stabilizes the release-ready vesicles by enhancing the interaction of the SNARE proteins with synaptotagmin during the priming process (Tian et al., 2005). Thus, dysbindin may modulate vesicle priming by its interaction with snapin.

Dysbindin is a subunit of biogenesis of lysosome-related organelle complex-1 (BLOC-1). We previously reported that the sdy mouse displays defects in lysosome-related organelles.
biogenesis (Li et al., 2003). Therefore, our observed alteration in vesicle structure may arise from deficiency-induced alteration in vesicle structure and reflect defective biogenesis of synaptic vesicles and LDCVs via a BLOC-1–dependent pathway. Besides, dysbindin also binds to the dystrophin–associated protein complex (DPC), a component of the synapse (Benson et al., 2001). DPC is required for proper maturation and function of a subset of inhibitory synapses because the cerebellar synapse is defective in mice lacking α- and β-dystrobrevin (Grady et al., 2006). This might explain the increased width of the synaptic cleft and increased thickness of PSDs in the sdy hippocampal asymmetrical synapse (Fig. 5). These morphological changes in the postsynaptic structure and synaptic cleft predict a change in postsynaptic function. However, our electrophysiological recordings show no changes at the postsynaptic site in sdy hippocampus (Figs. 4 B and S2). Future work is needed to address this puzzle.

Our results reveal that dysbindin deficiency produces a specific set of “dysbindin-related defects” in neurotransmitter release, including slow fusion kinetics, increased Q, decreased release probability, and smaller RRP. These abnormalities might cause an abnormal efficacy of synaptic transmission in sdy synapses. As it is generally proposed that schizophrenia is a disorder of synaptic transmission (Frankle et al., 2003; Harrison and Weinberger, 2005), our findings present the first description that part of the sdy endophenotypes of transmitter release might contribute to schizophrenia-like traits. However, further studies should be performed for whether what dysbindin-related defects in exocytosis lead to the cognitive impairments or psychotic symptoms seen in humans.

Schizophrenia is a heterogeneous syndrome with subtle behavioral and psychiatric abnormalities. Genetic variation of dysbindin has been associated with negative symptoms in patients with schizophrenia (DeRosse et al., 2006). These negative symptoms are difficult to identify in mice with any known diagnostic laboratory tests (Ross et al., 2006). Therefore, although our initial behavioral observations, including prepulse inhibition, did not show differences between sdy and WT mice (Li et al., 2003), more elaborate tests need to be performed to see whether sdy mice have any equivalent of psychotic symptoms. Nevertheless, our findings have indicated that the sdy mutant, originally identified as a model of Hermansky-Pudlak syndrome type 7 (Li et al., 2003), may also be useful for studying the pathogenesis of schizophrenia, particularly in the area of neurotransmitter release.

Materials and methods

Animals and slice preparations

The sdy mutant and control DBA/2j mice (WT) were originally obtained from the Jackson Laboratory, transferred from R.T. Swank’s laboratory at the Roswell Park Cancer Institute (Buffalo, NY) and bred in the animal facility of the Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences. All procedures were approved by the Institutional Animal Care and Use Committee (mouse protocol KY2005D-004) and the Peking University Committee on Ethics in the Care and Use of Laboratory Animals. To ensure the genotypes of sdy mice and were immediately immersed in ice-cold, low Ca2+ bicarbonate-buffered saline (BBS) containing 125 mM NaCl, 2.5 mM KCl, 0.1 mM CaCl2, 5 mM MgCl2, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose, pH 7.4, when gassed with 95% O2/5% CO2. After that, a single gland was glued with cyanoacrylate to the stage of a vibratome chamber and covered with the same cold, O2-saturated BBS. Slices (100–200 μm) were cut parallel to the larger base of the gland (Vibratome 1000, Vibratome). They were then incubated for 30 min at room temperature in normal BBS containing 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose gassed with 95% O2/5% CO2. Slices could be used for up to 8 h after cutting. For amperometric measurements and whole-cell recordings, slices were transferred to a recording chamber attached to the stage of an upright microscope equipped with an infrared-sensitive charge-coupled device camera (U-MPlanFl/IR, 60X, Olympus) and continuously superfused with normal BSS at room temperature.

Coronal hippocampal slices with a thickness of 300 μm from 12-d-old WT or sdy mice were made in ice-cold low-Ca2+ slicing solution containing 125 mM NaCl, 2.5 mM KCl, 0.1 mM CaCl2, 5 mM MgCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, 0.4 mM L-ascorbic acid, and 12.5 mM glucose, pH 7.4, when gassed with 95% O2/5% CO2. The slices were incubated for 1 h at room temperature in standard oxygenated extracellular solution containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1.3 mM MgCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, 0.4 mM L-ascorbic acid, and 12.5 mM glucose, pH 7.4.

Patch clamp recordings and amperometry in adrenal slices

We performed whole-cell recordings in adrenal slices using an EPC9/2 amplifier and Pulse software (HEKA Elektronik). The intracellular pipette solution contained 145 mM CsCl, 8 mM NaCl, 1 mM MgCl2, 10 mM H-Trisphes, 2 mM Mg2ATP, and 0.2 mM Na2GTP, pH 7.2, adjusted with CsOH. For experiments using intracellular dialysis of dysbindin peptide, the internal solution contained dysbindin or heat-denatured dysbindin. Recordings were made at room temperature.

Highly sensitive and low-noise 5-μm carbon fiber electrodes (CFEs; ProCFS; Dagan) were used to measure quantum release of catecholamines from chromaffin cells. The amperometric current (Iamp) was measured at a holding potential of 780 mV. Amperometric signals were low-pass filtered at 12 kHz and digitized at 1 kHz. The CFE surface was positively charged in contact with the membrane of a clean cell, and the close proximity of the electrode surface to the cell surface was confirmed by a slight deformation in the outline of the cell. High-K+ solution, one secretagogue used in our experiments, was applied using a perfusion system with a fast exchange time (PCR-2B, Instituto Nacional de Biodiversidad).

For analysis of the kinetic properties of amperometric spikes (Fig. 1 A), including the HHD, RT, and Q, only events >5 SD of the noise were used (Zhou and Misler, 1996). Data analysis was performed using Igor software (WaveMatrix with a custom-made macro program [Zhou and Misler, 1996]).

Miniature and evoked EPSC recording in hippocampal slices

mEPSCs were recorded in ventral hippocampal CA1 pyramidal neurons under a voltage clamp using an EPC-9 amplifier as described previously (Huang et al., 2007). The intracellular pipette solution contained 120 mM cesium-gluconate, 20 mM CsCl, 0.3 mM CaCl2, 4 mM NaCl, 0.5 mM EGTA, 10 mM H-Trisphes, 0.3 mM Na2GTP, 14 mM phosphocreatine, and 4 mM Mg2ATP, pH 7.2, with CsOH. 1 μM tetrodotoxin and 10 μM bicusculine were added to the external solution. Each individual mEPSC event was fully characterized by the parameters of amplitude, frequency, HHD, charge, RT, and decay time. These parameters were analyzed using the MiniAnalysis program (Synaptosoft, Inc.). Averaged mEPSC waveforms were obtained by averaging 50 individual events using Igor software.

Evoked EPSCs were elicited by electrical stimulation of the Schaffer collaterals with a bipolar silver electrode placed in the stratum radiatum (~150 μm away from the recorded CA1 pyramidal neuron. A single pulse (0.5 ms at 50 μA) was generated by a Grass stimulator (S88; Grass Technologies) and delivered through an isolation unit. EPSCs were recorded at ~70 mV in the presence of 10 μM bicusculine. EPSC amplitudes were determined by subtracting the baseline current (obtained by averaging the period 3 ms before the stimulus) from the peak current of the EPSC. Kinetic analyses (charge, RT, and decay time) were performed using Igor software with a custom-made macro program.

Purification of dysbindin

We amplified mouse full-length dysbindin cDNA (isoform 1, RefSeq available from GenBank/EMBL/DDJB) under accession no. NM_025772) by RT-PCR from DBA/2j total brain RNA with the introduction of NdeI and XhoI restriction sites at the 5‘ and 3‘ ends, respectively. The PCR product

Schizophrenia is a heterogeneous syndrome with subtle behavioral and psychiatric abnormalities. Genetic variation of dysbindin has been associated with negative symptoms in patients with schizophrenia (DeRosse et al., 2006). These negative symptoms are difficult to identify in mice with any known diagnostic laboratory tests (Ross et al., 2006). Therefore, although our initial behavioral observations, including prepulse inhibition, did not show differences between sdy and WT mice (Li et al., 2003), more elaborate tests need to be performed to see whether sdy mice have any equivalent of psychotic symptoms. Nevertheless, our findings have indicated that the sdy mutant, originally identified as a model of Hermansky-Pudlak syndrome type 7 (Li et al., 2003), may also be useful for studying the pathogenesis of schizophrenia, particularly in the area of neurotransmitter release.
was purified with the Gel Extraction mini kit (Watson Biotechnologies, Inc.), cloned into a pGEM T Easy vector (Promega), and verified by sequencing. We then subcloned the DNA fragment into the NdeI–Xhol–cleaved plasmid p28 (a gift from W. Gong, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). The p28-dysbindin construct was expressed in Escherichia coli Rosetta (DE3). The recombinant protein contains six histidine residues at the N terminus. The cells were harvested by centrifugation and resuspended in a lysis buffer containing 20 mM Tris-HCl, pH 8.0. The cell lysate was loaded on a nickel–nitrilotriacetic acid column (Qiagen), and His-tagged dysbindin was eluted with 0.5 M imidazole. We further purified it by gel filtration on a Superdex 200 column (GE Healthcare). The peak fractions were tested for purity by SDS-PAGE (Fig. S1) and the protein solution was exchanged using a centrifugal filter device (Amicon Ultra-4; Millipore). The dysbindin concentration was determined by a protein assay (Bio-Rad Laboratories).

Electron microscopy
Electron microscopy was performed as described previously (Huang et al., 2007), with a slight modification. In brief, for chromaffin cells, adrenal glands were removed from anesthetized 8-week-old WT and ady mice and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The samples were then treated with 1% OsO4 in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C, and serially dehydrated in ethanol followed by flat-embedding in Epon 812 and polymerization at 60°C for 48 h. Ultrathin sections (50–60 nm) were stained with uranyl acetate and lead citrate. The sections were examined on an electron microscope (JEM-1230, JEOL Ltd.) at 100 kV, and digital images were captured with a charge-coupled device camera system and analyzed with Image J software (National Institutes of Health). The number, size and spatial distribution of LDCVs were measured in sections of randomly selected cells from three different animals for both genotypes. The same single sections were analyzed at least twice independently, and the results were essentially identical. The quantitative analysis was performed as described previously (Colliver et al., 2000; Voets et al., 2001; Grabner et al., 2005). The area of each vesicle was measured and the diameter was calculated from the vesicle area using the equation: diameter = 2 × (area/π)0.5. The mean values were compared using a Student’s t test (SPSS 10.0, SPSS) and are displayed in Fig. 3.

For hippocampal electron microscopy examination, 8-week-old mice (three WT and three ady) under deep pentobarbital anesthesia were perfused through the heart with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and the hippocampus was sliced transversely to its longitudinal axis at 1 mm thickness. The blocks were trimmed to contain the hippocampal CA1 pyramidal cell bodies and their apical dendrites in the stratum radiatum. The blocks were immersed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C and the procedure described here was followed. Only asymmetrical, i.e., glutamatergic, synapses with clearly identifiable PSDs were analyzed. The quantitative analysis was performed according to previously published literature (Pozzo-Miller et al., 1999) and is displayed in Fig. 5.

Immunoblotting
Hippocampal and adrenal glands from ady mice were homogenized in a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and a protein inhibitor cocktail (Sigma-Aldrich). The extracts were centrifuged at 15,000 × g for 30 min and the concentration of the solubilized protein was determined by a protein assay (Bio-Rad Laboratories). For detection of various proteins in tissue homogenates, equal amounts of protein was determined by a protein assay (Bio-Rad Laboratories). Aliquots of protein were resolved by SDS-PAGE (Fig. S1) and the protein solution was exchanged using a centrifugal filter device (Amicon Ultra-4; Millipore). The dysbindin concentration was determined by a protein assay (Bio-Rad Laboratories).

Antibodies
Rabbit polyclonal dysbindin antibody (1:200) was a gift from D.J. Blake (University of Oxford, Oxford, UK). The mouse monoclonal antibodies against SNAP25 (1:500), syntaxin 1 (1:500), and dynamin I (1:500), and rabbit polyclonal anti-complexin I (1:500) were obtained from Santa Cruz Biotechnology, Inc. Other antibodies used in this study included monoclonal mouse anti-synaptogamin I (1:500; Assay Designs), rabbit polyclonal anti-VAMP2 (1:1,000; AbCam), mouse monoclonal anti-munc18-1 (1:1,000, BD Biosciences), mouse monoclonal VGLUT1 (1:500; Millipore), and mouse monoclonal anti-synaptobrevin I (1:500; Sigma-Aldrich).

All values throughout the text are presented as mean ± SEM. Either a Student’s t test or the Kolmogorov–Smirnov test was used to compare means when appropriate. Differences of P < 0.05 were considered statistically significant.

Online supplemental material
Fig. S1 shows the Coomassie blue staining of the purified recombinant dysbindin protein. Fig. S2 shows that the AMPA/NMDA ratio of evoked EPSCs is unaffected in sdy hippocampal neurons. Fig. S3 shows the measurements of RRP size and kinetics of replenishment of RRP in sdy and WT hippocampal neurons. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200711021/DC1.

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References


