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Dysbindin-containing complexes and their proposed functions in brain: from zero to (too) many in a decade

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ABSTRACT

Dysbindin (also known as dysbindin-1 or dystrobrevin-binding protein 1) was identified 10 years ago as a ubiquitously expressed protein of unknown function. In the following years, the protein and its encoding gene, *DTNBP1*, have become the focus of intensive research owing to genetic and histopathological evidence suggesting a potential role in the pathogenesis of schizophrenia. In this review, we discuss published results demonstrating that dysbindin function is required for normal physiology of the mammalian central nervous system. In tissues other than brain and in non-neuronal cell types, the protein has been characterized as a stable component of a multi-subunit complex, named BLOC-1 (biogenesis of lysosome-related organelles complex-1), which has been implicated in intracellular protein trafficking and the biogenesis of specialized organelles of the endosomal–lysosomal system. In the brain, however, dysbindin has been proposed to associate into multiple complexes with alternative binding partners, and to play a surprisingly wide variety of functions including transcriptional regulation, neurite and dendritic spine formation, synaptic vesicle biogenesis and exocytosis, and trafficking of glutamate and dopamine receptors. This puzzling array of molecular and functional properties ascribed to the dysbindin protein from brain underscores the need of further research aimed at ascertaining its biological significance in health and disease.

Key words: biogenesis of lysosome-related organelles complex-1 (BLOC-1), DTNBP1, dysbindin, dystrobrevin-binding protein, schizophrenia.

INTRODUCTION

Approx. 10 years ago, Benson et al. (2001) isolated, from murine brain and myotube cDNA libraries, multiple clones that were found to derive from a single gene and to encode a hitherto unknown protein; because the cDNA clones were isolated in a Y2H (yeast two-hybrid) screening for potential binding partners of β-dystrobrevin, the encoded protein was termed 'dysbindin' for 'dystrobrevin-binding protein'. The human and murine genes were then officially named *DTNBP1* (Entrez Gene ID: 84062) and *Dtnbp1* (Entrez Gene ID: 94245), respectively, both of them being short for 'dystrobrevin binding protein 1.' Although no other mammalian gene has been considered similar enough to deserve the term '*DTNBP2*' or the like, the products of two genes named (in humans) *DBNDD2* and *DBNDD1* have been occasionally referred to as 'dysbindin-2' and 'dysbindin-3,' respectively (Talbot et al., 2009). However, the sequence homology between the protein product of *DTNBP1* and those of *DBNDD1* and *DBNDD2* is restricted to less than half of the length of the former (Talbot et al., 2009; Cheli and Dell'Angelica, 2010). Consequently, it is pertinent to ask whether the products of *DBNDD1* and *DBNDD2* should be considered 'dysbindins' or, alternatively, proteins that share with the product of *DTNBP1* a conserved domain in the context of otherwise unrelated sequences. Consistent with this alternative view, the Human Genome Organization Gene Nomenclature Committee chose the names *DBNDD1* and *DBNDD2* as short for 'dysbindin (dystrobrevin binding protein 1) domain containing' 1 and 2. Therefore, there seems to be no compelling reason to change the name of dysbindin to 'dysbindin-1' or the like, and herein we will refer to this protein using its original name.

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Abbreviations: AP-3, adaptor protein-3; BLOC, biogenesis of lysosome-related organelles complex; coIP, co-immunoprecipitation; HEK-293 cells, human embryonic kidney cells; HPS, Hermansky–Pudlak syndrome; JNK, c-Jun N-terminal kinase; MS/MS, tandem mass spectrometry; RNAi, RNA interference; shRNA, short-hairpin RNA; siRNA, small-interfering RNA; WASH, Wiskott–Aldrich syndrome protein and SCAR homologue; VAMP-7, vesicle-associated membrane protein 7; WAVE, WASP (Wiskott–Aldrich syndrome protein) verprolin homologous; Y2H, yeast two-hybrid.

One year after publication of the first description of dysbindin, Straub et al. (2002) reported that allelic variants in *DTNBP1* were associated with an increased risk of developing schizophrenia among the members of 270 Irish families. This initial work, which was immediately followed by reports of positive association with the disease in other patient cohorts [reviewed by Benson et al. (2004a); Kendler (2004)], led to a flurry of studies aimed at establishing (i) the significance and molecular mechanism by which variations in *DTNBP1* would modify schizophrenia disease risk in the general population, (ii) the possible association between *DTNBP1* variants and other psychiatric disorders or cognitive functions and (iii) the biological plausibility of altered dysbindin function contributing to the pathogenesis of schizophrenia and related disorders. As of the beginning of 2011, over 260 articles could be found by searching the PubMed database with the combination of keywords 'dysbindin OR dtnbp1.'

The first two types of studies mentioned above (i and ii) have been discussed in recent reviews (Schwab and Wildenauer, 2009; Talbot et al., 2009). In short: large-scale genetic studies using a case-control design have failed to demonstrate genome-wide significance for any association between individual common variants in *DTNBP1* and schizophrenia in the general population of European ancestry or African–Americans (Sanders et al., 2008; Shi et al., 2009); although it should be noted that these studies have not been designed to explore potential genetic heterogeneity (Maher et al., 2010), epistatic interactions between variants in two or more genes (Edwards et al., 2008; Morris et al., 2008), interactions between genetic variants and environmental factors (Nicodemus et al., 2008) or the possibility that the genetic link between *DTNBP1* and the disease might be restricted to few families [reviewed by Psychiatric GWAS Consortium Steering Committee (2009)]. Nevertheless, decreased protein levels have been observed in hippocampus and prefrontal cortex of post-mortem brain samples from schizophrenic patients (Talbot et al., 2004; Tang et al., 2009a; Talbot et al., 2011), notably much more often than expected from the frequency of the allelic variants being considered as candidate risk factors of the disease. The evidence for genetic links between *DTNBP1* and other psychiatric disorders or neurobehavioural traits remains somewhat sparse, even though a recent meta-analysis provided support for an association between common variants in this gene and general cognitive ability in individuals with apparently no history of psychiatric disease (Zhang et al., 2010).

The third type of studies (iii), which is the main focus of this review, has uncovered multiple lines of evidence for important roles of dysbindin in brain. At first sight, these studies seem to provide strong support to the biological plausibility of *DTNBP1* influencing general cognitive ability and schizophrenia susceptibility. However, the devil lies in the details: the wide variety of biochemical and functional properties that have been ascribed to the dysbindin protein is striking, if not just perplexing. In this review, we discuss published evidence for (and in some cases against) the assembly of dysbindin into several multi-protein complexes with dissimilar properties as well as proposed roles of dysbindin and its associated complexes in multiple aspects of brain development and function.

BIOCHEMICAL PROPERTIES OF DYSBINDIN: A COMPLEX ISSUE

It is widely accepted that most proteins exert their biological functions in part through interaction with other proteins, thus providing a rationale for efforts to infer molecular functions from protein–protein interaction maps or 'interactomes' (von Mering et al., 2002). In the case of dysbindin, more than 140 binding partners have been described in the literature (Hikita et al., 2009; Oyama et al., 2009; Rodriguez-Fernandez and Dell'Angelica, 2009; Fei et al., 2010; Ito et al., 2010; Mead et al., 2010; Okuda et al., 2010). However, a few key issues deserve consideration.

First, owing to intrinsic limitations in the experimental methodologies, a significant fraction of the observed protein–protein interactions are likely to represent false positives, i.e. interactions that do not occur *in vivo* under physiological (or pathological) conditions. This is particularly problematic for interactions detected using the Y2H system, as the estimated false-discovery rate is of 50% or higher (Deane et al., 2002). Another methodology that is widely used to test for protein–protein interactions, namely coIP (co-immunoprecipitation) of pairs of epitope-tagged proteins following their simultaneous overexpression in cultured cells, is also prone to false positives. Even a method that is considered by many as the 'gold standard,' namely coIP of endogenously expressed proteins, can yield misleading results if not performed under carefully controlled conditions (Bonifacino and Dell'Angelica, 2001). Complementary evidence, such as co-localization at a subcellular level, is often used to argue in favour of the occurrence of protein–protein interactions *in vivo*. To this end, however, the subcellular distribution of dysbindin in brain cells will have to be defined more precisely, as several studies have reported localization to both presynaptic and postsynaptic terminals (Talbot et al., 2006; Ito et al., 2010), to axons, dendrites and soma (Taneichi-Kuroda et al., 2009; Ito et al., 2010) and to both cytoplasm and nucleus (Oyama et al., 2009; Fei et al., 2010).

Secondly, *in vivo* protein–protein interactions can be transient (e.g. lasting for milliseconds to seconds) or long lasting (lasting for hours to days) and biochemically stable. While both transient and long-lasting interactions may be physiologically significant, the concept of 'protein complex' and the experimental approaches used for their characterization may vary in important ways. Herein we use the expression 'stable protein complex' to describe multi-protein assemblies that have long half-lives (sometimes being considered 'permanent' as they assemble shortly after

synthesis of the subunits and last throughout the subunits' lifespan) and do not dissociate in the test tube unless subjected to strong denaturing conditions. It then follows that to demonstrate the existence of a stable protein complex one should seek more evidence than the simple demonstration of protein–protein interaction, especially in cases using sensitive methods that can also detect transient interactions (e.g. Y2H system, coIP of overexpressed proteins, etc.). For example, provided that appropriate reagents (e.g. specific antibodies) are available, one should be able to detect robust coIP of the endogenously expressed proteins and obtain evidence of biochemical co-fractionation (e.g. the proteins co-eluting upon size-exclusion chromatography or other forms of protein chromatography or co-migrating upon density gradient ultracentrifugation) under non-denaturing conditions. In addition, for 'permanent' stable protein complexes one may be able to observe that disruption of one subunit [in mutant model organisms or in cultured cells subjected to RNAi (RNA interference)] results in reduced protein levels of the others at steady state, as impaired assembly of such a complex would lead to unassembled subunits that are incorrectly folded and, hence, biochemically unstable and targeted for rapid intracellular degradation.

Thirdly, at least three dysbindin isoforms encoded by alternatively spliced transcripts have been detected in the human brain (Talbot et al., 2004; Oyama et al., 2009; Tang et al., 2009a; Ito et al., 2010), thus raising the possibility that the different protein isoforms might interact with different partners and even assemble into different protein complexes. It should be noted, however, that the vast majority of protein–protein interactions documented for dysbindin involved the longest isoform, which comprises slightly over 350 amino acid residues (351 in humans and 352 in mice) and migrates on SDS/PAGE protein gels with an electrophoretic mobility comparable with that of a 50-kDa polypeptide.

Finally, consistent with the focus of this review being on possible roles of dysbindin in the central nervous system, emphasis is herein given to interactions that have been documented for proteins endogenously expressed in the brain (Figure 1, black lines).

Is there any significant pool of monomeric dysbindin in the brain?

Given the large number of binding partners reported for dysbindin, one possibility to consider would be that dysbindin might exist in brain as a stable monomer (or homo-oligomer) that transiently engages different binding partners depending on intracellular localization and/or physiological condition. However, to our knowledge no biochemical evidence in support of such a possibility has been reported in the literature. On the contrary, on fractionation by size-exclusion chromatography of a soluble extract prepared from cerebral cortex and containing ~90% of all detectable dysbindin immunoreactivity, the elution profile consisted of a single peak corresponding to a Stokes radius of ~95 Å (i.e. larger than that of a large globular protein such as thyroglobulin) and no dysbindin immunoreactivity was detected in fractions corresponding to the range of hydrodynamic radii predicted for a dysbindin monomer (23–45 Å depending on its molecular shape) or a homo-dimer (28–57 Å) (Ghiani et al., 2010). Granted, this approach did not allow analysis of the ~10% of dysbindin immunoreactivity that remained in the insoluble fraction, but the results at the very least negate the existence of a significant pool of monomeric dysbindin in soluble form. It is worth mentioning that similar results had been previously obtained for dysbindin from liver (Li et al., 2003; Starcevic and Dell'Angelica, 2004). Furthermore, published observations of drastically reduced steady-state levels of dysbindin protein in brain (and other tissues) from mice carrying mutations in subunits of BLOC-1 (biogenesis of lysosome-related organelles complex-1) can be taken as evidence not only of a stable association of dysbindin into that complex (see below) but also of biochemical instability of monomeric (or homo-oligomeric) forms of dysbindin *in vivo*.

Does dysbindin assemble into the dystrophin–glycoprotein complex?

As mentioned in the Introduction, dysbindin was first characterized as a binding partner of α- and β-dystrobrevins (Benson et al., 2001). Both dystrobrevins display significant sequence homology to each other and to the C-terminal region of dystrophin, which is the product of the gene mutated in Duchenne and Becker muscular dystrophies. Dystrophin and its ubiquitously expressed, relatively shorter isoforms (e.g. Dp71) assemble into stable, membrane-associated complexes collectively known as the 'dystrophin–glycoprotein complex' or 'dystrophin-associated protein complex' (Blake et al., 2002; Durbeej and Campbell, 2002). In differentiated myotubes and glial cells, the complexes contain α-dystrobrevin, while in neurons they mainly contain the β-dystrobrevin paralogue (Blake et al., 1999). Having isolated dysbindin as a dystrobrevin-binding protein, it was pertinent to ask whether the former would be a stable component of a dystrophin-containing complex. However, a positive coIP between endogenous dysbindin and the dystrobrevins from brain and muscle (Benson et al., 2001) has later been called into question (Nazarian et al., 2006). While it is fair to state that the possibility of dysbindin being associated with the dystrobrevins within a dystrophin-containing complex has not been completely ruled out, new positive evidence would be necessary if one were to argue again in its favour (Figure 1, red line and question mark).

BLOC-1

BLOC-1 was initially described as a ubiquitously expressed, stable protein complex containing pallidin and muted, two small proteins encoded by the murine genes disrupted by the spontaneous mutations 'pallid' and 'muted' respectively

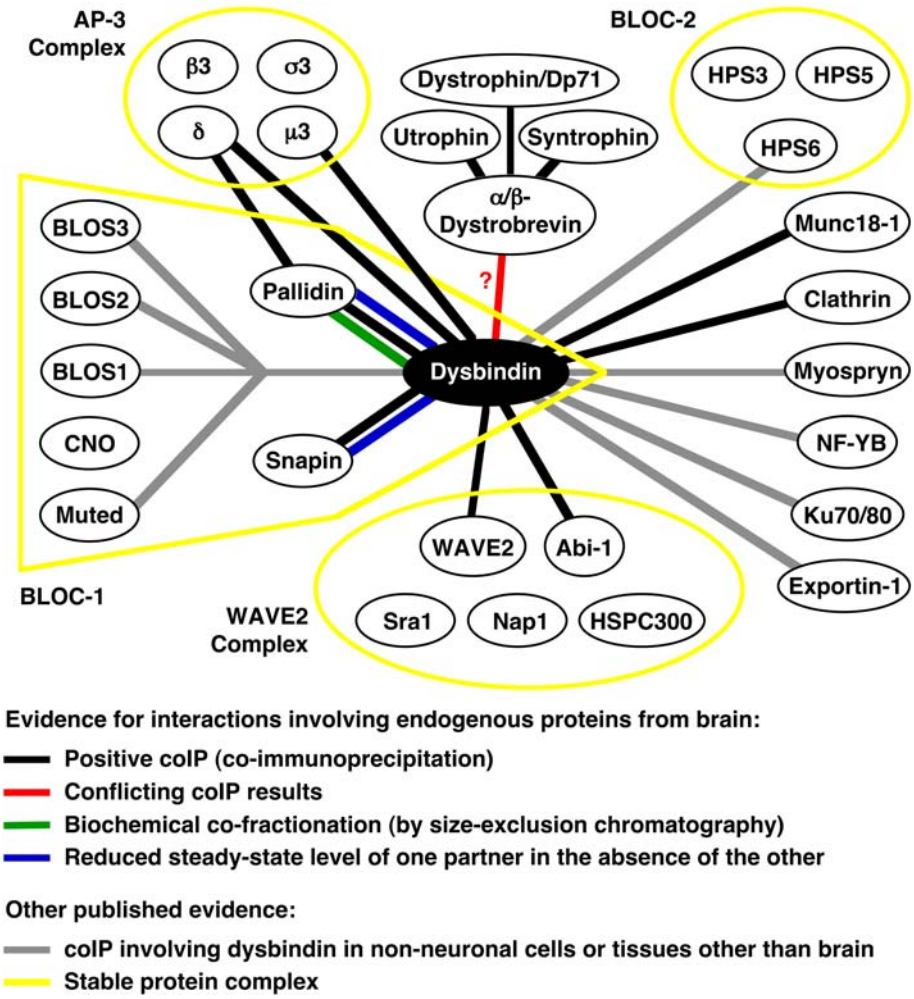

Figure 1 **Summary of published evidence in support of stable physical associations between endogenously expressed dysbindin and multiple interacting partners**
Interactions reported for dysbindin from tissues other than brain, or from non-neuronal cell types, are denoted in grey.

(Falcón-Pérez et al., 2002; Moriyama and Bonifacino, 2002). Mice homozygous for either pallid or muted mutations display reduced pigmentation of coat and eyes as well as extended bleeding times; these phenotypes are due to impaired formation of the lysosome-related organelles where melanin pigments are synthesized (i.e. the melanosomes) and of those where serotonin and other small-molecule activators of platelet aggregation are stored within circulating platelets (i.e. the platelet dense granules), respectively [reviewed by Raposo et al. (2007)]. These and other mutant murine strains had been postulated to serve as animal models of HPS (Hermansky–Pudlak syndrome), which is a rare genetic disorder characterized by defective biogenesis of melanosomes, platelet dense granules and other lysosome-related organelles (Swank et al., 1998). On realization that BLOC-1 should contain additional subunits besides pallidin and muted (Falcón-Pérez et al., 2002; Moriyama and Bonifacino, 2002), a logical next step was to search for them among the products of genes mutated in other murine models of the same disease. This approach led to the identification of cappuccino (Ciciotte et al., 2003) and dysbindin (Li et al., 2003) as BLOC-1 subunits.

The case of dysbindin deserves further consideration. The murine spontaneous mutation 'sandy' (Swank et al., 1991) was identified using a positional-cloning approach as an in-frame deletion in the *Dtnbp1* gene (Li et al., 2003). Consistent with the idea that the homozygous sandy mouse could serve as an animal model of HPS (Swank et al., 1991), mutation analyses following a candidate approach identified one patient suffering from that disease and carrying a non-sense mutation in *DTNBP1* [Li et al. (2003); so far, this patient has been the only human reported in the literature to carry a disease-causing mutation in this gene]. In addition, the same authors observed that the steady-state levels of the dysbindin protein were significantly reduced in kidney extracts prepared from homozygous pallid and muted mice, and conversely that those of pallidin and muted were decreased in kidney extracts from homozygous sandy mice. In addition, biochemical

co-fractionation between dysbindin and pallidin from liver cytosol was observed on size-exclusion chromatography and density-gradient ultracentrifugation (Li et al., 2003). These observations suggested that dysbindin is a stable component of BLOC-1, at least in tissues such as kidney and liver. Further evidence in support of this idea stemmed from: (i) partial purification of BLOC-1 from bovine liver, whereby comparable enrichment of dysbindin and pallidin was observed through several purification steps, (ii) identification of peptides derived from dysbindin, pallidin and other subunits by MS/MS (tandem mass spectrometry) of the purified complex, and (iii) coIP of endogenously expressed proteins from HeLa cells, whereby dysbindin co-immunoprecipitated not only with pallidin and muted but also with four additional subunits (snapin, BLOS1, BLOS2 and BLOS3) that had been identified by the same MS/MS analysis of purified BLOC-1 (Starcevic and Dell'Angelica, 2004).

Does dysbindin exist as a stable component of BLOC-1 also in the brain? The following lines of evidence argue in favour of this idea: first, coIP of endogenous proteins from murine brain was documented for dysbindin and pallidin (Nazarian et al., 2006) as well as for dysbindin and snapin (Talbot et al., 2006). Secondly, dysbindin co-fractionated with pallidin on size-exclusion chromatography of cytosol prepared from murine cerebral cortex (Ghiani et al., 2010). In fact, the Stokes radius estimated for the soluble form of brain dysbindin (Ghiani et al., 2010) is in close agreement with that estimated for BLOC-1 from murine liver cytosol (Li et al., 2003; Starcevic and Dell'Angelica, 2004). Finally, the steady-state protein levels of snapin were decreased in hippocampus from sandy mice (Feng et al., 2008), implying that dysbindin is important for biochemical stability of the snapin protein *in vivo*, and those of dysbindin were drastically reduced in hippocampus, cortex and cerebellum from pallid mice (Ghiani et al., 2010), implying that the stability of the dysbindin protein in turn depends on pallidin. Together, these observations make a strong case for the bulk of brain dysbindin existing as a stable component of BLOC-1 (Figure 1). However, two important issues should be borne in mind. The first issue concerns the possible existence in brain of a pool of dysbindin not associated with BLOC-1, for example, associated with an alternative protein complex. In our opinion, the published biochemical data do not rule out this possibility, although they indicate that if such a pool indeed existed it would represent a small fraction of the dysbindin protein that is present in brain at steady state. The second issue concerns the exact subunit composition of BLOC-1 in the central nervous system. Here, the published results indicate that dysbindin is associated with a stable complex with pallidin and snapin in the brain; however, for the remaining subunits all published evidence for their stable association into BLOC-1 has been obtained in tissues other than brain or in non-neuronal cell types (Figure 1). Hence, the possibility that BLOC-1 from brain and from other tissues could differ in subunit composition, for instance, due to the existence of brain-specific subunits, deserves future investigation.

Two recent studies have raised the notion that BLOC-1 might contain additional subunits besides those depicted in Figure 1, at least in certain cell types. In the first study, an actin nucleation-promoting factor named WASH (for 'Wiskott-Aldrich syndrome protein and SCAR homologue') was proposed to be a component of BLOC-1 (Monfregola et al., 2010). However, the published biochemical data (i.e. coIP of WASH with overexpressed BLOS2, *in vitro* interaction between WASH and a recombinant form of BLOC-1) do not allow discrimination between WASH being a stable subunit of BLOC-1 and being a transient-binding partner of the complex. It should be noted that WASH has recently been reported to assemble into a large stable protein complex (Derivery et al., 2009; Gomez and Billadeau, 2009), with an estimated molecular mass (Derivery et al., 2009) that is more than twice that estimated for BLOC-1 (Falcón-Pérez et al., 2002). Moreover, the WASH complex has been recently purified from bovine brain and found to contain five subunits, namely WASH plus the proteins FAM21/VPEF, Strumpellin, CCDC53 and SWIP/KIAA1033 (Jia et al., 2010), all of which are distinct from the eight known subunits of BLOC-1. The second study consisted of a phylogenetic analysis combined with mining of interactomics data, which led the authors to postulate that an uncharacterized protein encoded by the human gene *C19orf50* (Entrez Gene ID: 79036) might represent a novel BLOC-1 subunit (Hayes et al., 2011). Although this protein has not been detected by MS/MS of partially purified BLOC-1 from bovine liver (Starcevic and Dell'Angelica, 2004) or of BLOC-1 subunits and other interacting proteins immunoprecipitated from HEK-293 cells (human embryonic kidney cells) expressing an epitope-tagged form of dysbindin (Mead et al., 2010), the possibility that the *C19orf50* gene product might be part of BLOC-1 in other cell types – including those of the central nervous system – remains to be addressed experimentally.

WAVE2 [WASP (Wiskott–Aldrich syndrome protein) verprolin homologous 2] complex

In addition to being described as a component of BLOC-1, dysbindin from the brain has been proposed to form a ternary complex with an actin nucleation-promoting factor named WAVE2 and its interacting partner Abi-1 (Ito et al., 2010). Different WAVE complexes are formed by combinations of subunit isoforms encoded by paralogous genes, including WAVE1, WAVE2 and WAVE3 as well as Abi-1, -2 and -3 (Veltman and Insall, 2010). So far, the published biochemical evidence for the existence of a ternary dysbindin/WAVE2/Abi-1 complex is limited to coIP experiments, namely those between endogenous dysbindin and WAVE2 (or WAVE1, as the polyclonal antibody recognized both WAVE paralogues but not WAVE3) as well as the former and Abi-1 from rat brain (Ito et al., 2010). Stable protein complexes containing WAVE1 and WAVE2 were purified from bovine brain and non-neuronal cell lines, respectively, and several subunits were identified by MS/MS (Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004). In particular, the WAVE2 complex isolated from human HeLa cells (Gautreau et al., 2004) was

found to contain Abi-1, Sra1, Nap1 and HSPC300 (Figure 1) but no dysbindin. In light of these findings, it seems tempting to speculate that the published coIP between endogenous dysbindin and WAVE2 (or WAVE1) as well as between the former and Abi-1 (Ito et al., 2010) might reflect an interaction (stable enough to allow detection by coIP) between BLOC-1 and a WAVE complex in brain. Intriguingly, the WAVE proteins are structurally related to WASH (Veltman and Insall, 2010), which as mentioned above has been reported to interact with known BLOC-1 subunits (Monfregola et al., 2010).

Other interacting partners

In addition to the aforementioned protein complexes into which dysbindin has been proposed to assemble, several protein–protein interactions involving dysbindin and stable enough to enable detection by coIP of endogenously expressed proteins have been reported in the literature (Figure 1). More transient interactions have been analysed and discussed in recent publications (Rodriguez-Fernandez and Dell'Angelica, 2009; Mead et al., 2010).

The AP-3 (adaptor protein-3) complex, clathrin, and Munc18-1, are known components of the cellular machinery that mediates intracellular trafficking of membrane lipids and proteins. The AP-3 complex and clathrin participate in membrane budding at the 'origin' of various trafficking routes, with AP-3 acting primarily on endosomes and serving as a sorting device that recruits the protein cargo [reviewed by Dell'Angelica (2009)] and clathrin acting on multiple cellular locations and serving as a 'scaffold' that facilitates protein-driven membrane deformation [reviewed by Hurley et al. (2010)]. Munc18-1 belongs to a family of proteins that regulate membrane fusion; in particular, Munc18-1 is expressed mainly in neurons and neuroendocrine cells and regulates exocytosis of synaptic and dense core vesicles [reviewed by Han et al. (2010)]. Interactions between endogenous dysbindin and AP-3 (Taneichi-Kuroda et al., 2009; Hikita et al., 2009; Newell-Litwa et al., 2010), clathrin (Hikita et al., 2009), and Munc18-1 (Hikita et al., 2009), were all observed by coIP of rodent brain extracts. CoIP between endogenously expressed dysbindin and AP-3 was also documented using undifferentiated rat pheochromocytoma PC12 cells, although the interaction was somewhat labile and only observed on covalent stabilization with a chemical cross-linker (Newell-Litwa et al., 2010).

The interaction between dysbindin and the AP-3 complex had been previously observed by coIP using HeLa cells and murine liver (Di Pietro et al., 2006) and by immunoisolation from PC12 cells of AP-3 and binding partners (the latter stabilized by chemical cross-linking) followed by MS/MS analysis (Salazar et al., 2009). More recently, it has been detected by MS/MS analysis of dysbindin-interacting proteins immunoprecipitated from HEK-293 cells expressing an epitope-tagged form of dysbindin (Mead et al., 2010). As in the case of BLOC-1, recessive mutations in the gene encoding a subunit of AP-3 (the *AP3B1* gene encoding the β3A subunit) cause HPS in a small subset of the patients suffering from this disease [reviewed by Huizing et al. (2008)]. Besides the ubiquitously expressed AP-3, a second form of the complex containing alternative β3 and μ3 subunits (in humans encoded by the *AP3B2* and *AP3M2* genes) exists in brain [reviewed by Newell-Litwa et al. (2007)] and may interact with dysbindin as well (Hikita et al., 2009; Oyama et al., 2009). Importantly, at least three lines of evidence suggest that interaction with AP-3 occurs *in vivo* in the context of dysbindin assembled into BLOC-1. First, coIP between endogenous dysbindin and AP-3 from liver required an intact BLOC-1, as the coIP was negative when tested under identical conditions using liver extracts from pallid mutant mice (Di Pietro et al., 2006). Secondly, other BLOC-1 subunits besides dysbindin, namely pallidin, muted, cappuccino and BLOS3, were detected in a purified preparation of cross-linked AP-3-binding partners from PC12 cells (Salazar et al., 2009). Thirdly, coIP between the AP-3 complex and a second BLOC-1 subunit, pallidin, has been observed using murine neocortical neurons (Newell-Litwa et al., 2009) and a synaptosome-enriched fraction from rat brain (Newell-Litwa et al., 2010).

BLOC-2 is a stable protein complex that, like AP-3 and BLOC-1, has been implicated in intracellular protein trafficking and contains as subunits the products of genes mutated in various forms of HPS [reviewed by Raposo et al. (2007); Huizing et al. (2008)]. Interaction between dysbindin and the HPS6 subunit of BLOC-2 has been detected by coIP using HeLa cell extracts and deemed to reflect a physical association between the two intact complexes, BLOC-1 and -2 (Di Pietro et al., 2006).

Myospryn (cardiomyopathy-associated protein 5, *CMYA5* gene product) is a large (~4000 amino acid residues) protein that belongs to the superfamily of TRIM (for 'tripartite motif') proteins. It was identified in a Y2H screening for potential binding partners of dysbindin; interaction between endogenously expressed dysbindin and myospryn was demonstrated by coIP using murine skeletal muscle (Benson et al., 2004b). Although the expression of myospryn is highly restricted to skeletal and cardiac muscle (Benson et al., 2004b), and the protein has been linked to muscular dystrophy and to left ventricle abnormalities [reviewed by Sarparanta (2008)], intriguingly a recent study has proposed that *CMYA5* should be considered a schizophrenia risk gene (Chen et al., 2011). Therefore, future attempts to detect the myospryn protein and its potential interaction with dysbindin in brain seem warranted.

Finally, a small pool of dysbindin immunoreactivity has been reported to localize at steady state inside the nuclei of neuroblastoma SH-SY5Y (Oyama et al., 2009) and N2a (Fei et al., 2010) cells but not of MNT-1 melanoma cells (Di Pietro et al., 2006) or primary rat hippocampal neurons (Fei et al., 2010). In addition, Fei et al. (2010) reported the presence in dysbindin of a nuclear export signal, which was functional in the context of an epitope-tagged dysbindin construct.

Consistent with the notion that dysbindin would shuttle in and out of the nucleus, coIP between dysbindin and exportin-1, a well-known nucleocytoplasmic transport factor involved in nuclear export (Cook et al., 2007), was observed for the endogenous proteins from N2a cells (Fei et al., 2010). Also consistent with the existence of a nuclear pool of dysbindin are the reports of coIP between this protein and Ku70 and Ku80 (Oyama et al., 2009), which are both components of the DNA-dependent protein kinase complex involved in the repair of DNA double-strand breaks [reviewed by Collis et al. (2005)], as well as between the former and the nuclear transcription factor NF-YB (nuclear factor-YB; Okuda et al., 2010). The potential for dysbindin to exert some biological functions inside the nucleus deserves further investigation.

ANIMAL MODELS OF DYSBINDIN DEFICIENCY

Several significant advances in our understanding of the possible physiological roles of dysbindin in brain have stemmed from studies using the sandy mouse as an animal model of dysbindin deficiency. In the following sections, we summarize published evidence indicating that altered dysbindin function can lead to abnormalities in animal behaviour and synaptic transmission, and discuss the need for additional animal models besides sandy.

The 'sandy' mouse: a background check

Following the initial reports suggesting the existence of a genetic association between *DTNBP1* variants and schizophrenia [reviewed by Benson et al. (2004a); Kendler (2004)] and the molecular identification of the sandy allele (now referred to as *Dtnbp1*^{sdy}) as an internal deletion in the murine dysbindin-encoding gene (Li et al., 2003), several groups sought to determine whether mice carrying this allele would display, besides the pigmentation and platelet- and kidney-related defects previously reported in the literature (Swank et al., 1991), any phenotype that would be indicative of an important physiological function of dysbindin in brain. As the *Dtnbp1*^{sdy} allele had arisen by spontaneous mutation in a stock of the DBA/2J strain (Swank et al., 1991), initial studies were carried out in the context of this genetic background; subsequently, three groups have transferred the allele into the C57BL/6J background (reviewed by Talbot, 2009; see also Ji et al., 2009; Tang et al., 2009b; Karlsgodt et al., 2011; Papaleo et al., 2011). All in all, these research efforts have resulted in a flurry of papers documenting multiple behavioural and electrophysiological abnormalities displayed by sandy mice (Tables 1 and 2). Although it seems fair to say that these findings are among the most exciting of those resulting from 10 years of research in dysbindin, two issues described below deserve special attention.

First, close inspection of the published results reveals some apparent inconsistencies that remain to be explained. In the case of the behavioural analyses (Table 1), the genetic background seems to have contributed to paradoxical effects of the *Dtnbp1*^{sdy} allele on locomotor activity and motor balance skill. Indeed, homozygous mutants in the DBA/2J background were reported to display, relative to DBA/2J controls, reduced locomotor activity in the open field test (Hattori et al., 2008; Takao et al., 2008) and impaired performance in the rotarod test (Takao et al., 2008), whereas in the C57BL/6J background the homozygous mutants displayed hyperactivity (Cox et al., 2009; Ji et al., 2009; Papaleo et al., 2011) and improved motor balance skills (Cox et al., 2009) in similar tests carried out using C57BL/6J mice as controls. In addition, different experimental conditions used in the behavioural tests could potentially explain apparent inconsistencies that cannot be ascribed to genetic background; for example, the reports of decreased (Hattori et al., 2008; Takao et al., 2008) and normal (Feng et al., 2008; Bhardwaj et al., 2009) locomotor activity displayed by DBA/2J mice homozygous for the *Dtnbp1*^{sdy} allele. These apparent inconsistencies notwithstanding, it is worth noting that impaired working memory has been consistently observed for homozygous mutant mice regardless of genetic background (Table 1). In the case of the electrophysiological analyses (Table 2), it is curious that the earlier studies using mice of the DBA/2J background have been interpreted in terms of dysbindin deficiency causing presynaptic abnormalities (Chen et al., 2008; Jentsch et al., 2009) and the most recent studies using mice of the C57BL/6J background have been interpreted in terms of dysbindin deficiency causing postsynaptic abnormalities (Ji et al., 2009; Tang et al., 2009b; Karlsgodt et al., 2011; Papaleo et al., 2011). At this point, it seems hard to distinguish between these differences reflecting effects due to genetic background or simply a shift in the focus of research. In any event, the possibility of impaired dysbindin function leading to alterations at both presynaptic and postsynaptic terminals (see below) merits consideration. Other differences between effects of the mutation on electrophysiological measurements probably reflect the fact that different neuronal cell types in different brain areas have been examined (Table 2; see also Papaleo and Weinberger, 2011).

Secondly, it is noteworthy that *Dtnbp1*^{sdy}, which was originally characterized as a recessive allele (Swank et al., 1991; Li et al., 2003), behaved in a dominant or semi-dominant fashion to elicit many of the behavioural and electrophysiological phenotypes for which both homozygous and heterozygous mutants have been tested (Tables 1 and 2). Granted, a simple and satisfactory explanation would be that these phenotypes reflect biological activities of dysbindin in neurons that, unlike those in non-neuronal cells, cannot be fulfilled by the protein levels achieved by expression from a single copy of the wild-type allele. Along these lines, partial reductions in dysbindin protein levels have been documented in synaptosomes prepared from brains of *Dtnbp1*^{sdy}/+ heterozygous mice (Talbot, 2009), although direct experimental evidence demonstrating that these intermediate levels are insufficient to sustain normal dysbindin function in

Table 1 **Behavioural abnormalities reported for sandy mice***

		Behavioural assay			
Genetic background	**Tested Dtnbp1 genotype(s)**	**Test**	**Drug or special condition**	**Observed abnormality**	**Reference**
DBA/2J	*sdy/sdy*	Open field	None	↓ Locomotor activity	Takao et al. (2008)
			0–15 min	↓ Locomotor activity	Hattori et al. (2008)
		Light/dark transition	None	↓ Locomotor activity; ↓ Exploratory behaviour	Takao et al. (2008)
		Elevated plus maze	None	↓ Number of arm entries	Hattori et al. (2008)
		Rotarod test	After four trials	↓ Motor balance skill	Takao et al. (2008)
		Social interaction	None	↓ Number and ↓ duration of social contacts	Hattori et al. (2008)
				↓ Duration of social contacts	Feng et al. (2008)
		Novel object recognition	None	↓ Novel object preference	Feng et al. (2008)
		Barnes circular maze	7 days after last training	↓ Memory retention	Takao et al. (2008)
		T-maze forced alternation	Sessions 11–15	↓ Working memory	Takao et al. (2008)
	sdy/sdy; sdy/+	Locomotor activity	Day 7 (trials on days 1–4)	↓ Habituation to environment (recessive)	Bhardwaj et al. (2009)
			AMPH (acute dose)	↓ Stimulation of locomotion (semi-dominant?)	Bhardwaj et al. (2009)
			AMPH (dose after chronic)	↑ Response in pre-treated group (recessive)	Bhardwaj et al. (2009)
		Novel object recognition	None	↓ Novel object preference (dominant)	Bhardwaj et al. (2009)
		Fear conditioning	None	↑ Freezing to conditioned stimulus (recessive)	Bhardwaj et al. (2009)
		Thermal nociception	None	Hypoalgesia (dominant)	Bhardwaj et al., (2009)
		Delayed non-match-to-position	None	↓ Spatial working memory (semi-dominant?)	Jentsch et al. (2009)
C57BL/6J	*sdy/sdy*	Open field	None	↑ Locomotor activity	Ji et al. (2009)
	sdy/sdy; sdy/+	Open field	None	↑ Locomotor activity (recessive); ↓ habituation (recessive)	Cox et al. (2009)
				↑ Locomotor activity (recessive)	Papaleo et al. (2011)
		Rotarod test	After four trials	↑ Motor balance skill (dominant)	Cox et al. (2009)
		Elevated zero maze	None	↑ Locomotor activity (semi-dominant?)	Cox et al. (2009)
		Morris water maze	Hidden platform	↑ Escape latency (recessive); ↓ preference for target quadrant (recessive)	Cox et al. (2009)
		Delayed non-match-to-position	None	↓ Spatial working memory (dominant)	Karlsgodt et al. (2011)
		T-maze task	None	Faster memory acquisition (semi-dominant)	Papaleo et al. (2011)
			↓ Inter-trial delay	↓ Working memory (dominant)	Papaleo et al. (2011)
			New cage stress	↓ Working memory (dominant)	Papaleo et al. (2011)
		Acoustic startle	120 dB	↑ Startle reactivity (recessive); ↑ prepulse inhibition (recessive)	Papaleo et al. (2011)
			+ D2 agonist (quinpirole)	↓ Startle reactivity (dominant?); ↓ prepulse inhibition (semi-dominant)	Papaleo et al. (2011)

*Behavioural tests were carried out using sandy (*Dtnbp1*^{sdy}) mice and 'wild-type' controls of equivalent genetic background. In experiments in which both heterozygous and homozygous mutants were analysed, the terms 'recessive,' 'dominant' and 'semi-dominant' denote that the heterozygotes behaved like the wild-type controls, like the homozygous mutants or displaying intermediate phenotypes, respectively. AMPH, amphetamine.

Table 2 **Electrophysiological abnormalities reported for sandy mice***

		Electrophysiology			
Genetic background	**Tested Dtnbp1 genotype(s)**	**Tissue region**	**Cell type**	**Observed abnormality**	**Reference**
DBA/2J	*sdy/sdy*	Adrenal medulla	Chromaffin cell	Slow release kinetics; ↑ quantal size; ↓ number of spikes per cell; ↓ total evoked current; ↓ RRP size	Chen et al. (2008)
		Hippocampus	Pyramidal neuron	↓ Frequency and ↑ quantal size of mEPSCs; ↓ peak amplitude and ↑ decay time of eEPSCs; ↓ RRP size	Chen et al. (2008)
			(Field recording)	↑ Serotonin-induced potentiation	Koboyashi et al. (2011)
	sdy/sdy; *sdy/+*	PFC	Pyramidal neuron	↓ Amplitude of eEPSCs (dominant); ↓ frequency of mEPSCs (dominant); ↓ amplitude of mEPSCs (semi-dominant?); ↓ paired-pulse facilitation (semi-dominant); ↓ rheobase and ↓ spike threshold (semi-dominant)	Jentsch et al. (2009)
C57BL/6J	*sdy/sdy*	PFC	Pyramidal neuron	↓ Frequency and ↓ amplitude of sIPSCs	Ji et al. (2009)
			Fast-spiking inter-neuron	↓ Number of spikes induced by depolarization	Ji et al. (2009)
		Medial PFC	Pyramidal neuron	↑ Number of spikes induced by depolarization; ↑ response to D2 agonist (quinpirole); ↑ frequency of sEPSCs	Papaleo et al. (2011)
			Fast-spiking inter-neuron	↓ Frequency of sEPSCs	Papaleo et al. (2011)
		Striatum	Fast-spiking interneuron	↓ Number of spikes induced by depolarization; ↑ response to D2 agonist (quinpirole)	Ji et al. (2009)
		Hippocampus	Pyramidal neuron	↑ Amplitude and ↓ decay time of NMDAR-mediated EPSCs (NR2B antagonist insensitive); ↑ NMDA/AMPA ratio	Tang et al. (2009b)
			(Field recording)	↑ Long-term potentiation	Tang et al. (2009b)
	sdy/sdy; *sdy/+*	Pre- or infra-limbic cortex	Pyramidal neuron	↓ Amplitude of NMDA-evoked current (dominant)	Karlsgodt et al. (2011)

*Electrophysiological recordings were carried out on tissue slices from sandy (*Dtnbp1*^{sdy}) mice and 'wild-type' controls of equivalent genetic background. In experiments in which both heterozygous and homozygous mutants were analysed, the terms 'dominant' and 'semi-dominant' are used to indicate that the abnormalities observed in samples from heterozygotes were as severe as those from homozygotes or of intermediate severity, respectively. EPSC, excitatory postsynaptic current; eEPSC, evoked EPSC; mEPSC, miniature EPSC; PFC, prefrontal cortex; RRP, readily releasable pool; sEPSC, spontaneous EPSC; sIPSC, spontaneous inhibitory postsynaptic current.

neurons is lacking. At least two alternative explanations cannot be ruled out at this point and, hence, deserve consideration. The first alternative is based on the facts that the *Dtnbp1*^{sdy} allele is, strictly speaking, not a null mutation but a partial deletion that comprises two internal exons and does not create frameshift in any predicted open reading frame, and that transcripts expressed from the mutant allele have been detected at levels comparable to those expressed from the wild-type allele (Li et al., 2003). Accordingly, one would expect abnormal dysbindin protein with an in-frame deletion (encompassing 52 residues) to be synthesized in cells carrying the *Dtnbp1*^{sdy} allele. Although originally it was assumed that the abnormal protein might be quickly degraded before it could exert any biological effect, its exact fate in neurons (where alternatively spliced forms are expressed) remains unknown and, in light of the behaviour of *Dtnbp1*^{sdy} as a semi-dominant or dominant allele for certain behavioural or electrophysiological phenotypes, should be addressed experimentally. The second alternative explanation, albeit merely hypothetical, is that a second-site mutation in a neighbouring gene might be responsible for some of the observed phenotypes. In principle, these alternative explanations could be addressed by phenotype-rescue experiments in mice carrying the mutant allele and transgenic BAC clones encompassing the wild-type *Dtnbp1* gene (similar to what was done to verify that the pigmentation and platelet-related phenotypes of sandy mice are a consequence of dysbindin deficiency; Li et al., 2003) or by analysis of mice carrying independent mutant alleles (see below).

Other murine lines of interest

A second mutant allele of the murine dysbindin-encoding gene, named 'salt and pepper' or 'sandy Bruce Beutler' and

currently referred to as *Dtnbp1*$^{sdy-Btlr}$, has been obtained by chemical mutagenesis of C57BL/6J mice (Blasius et al., 2010). The mutation consists of a single nucleotide change in a donor splice site and is predicted to result in skipping of exon 5, with subsequent frameshift and early termination of translation. However, skipping of the exon may be partial, at least in certain cell types, as the pigmentation phenotype of homozygous *Dtnbp1*$^{sdy-Btlr}$ mice (see http://mutagenetix.scripps.edu) is significantly less severe than that of homozygous *Dtnbp1*^{sdy} mice in the same background. Other alleles of *Dtnbp1* are currently being generated by large-scale gene-trapping efforts; in particular, the European Conditional Mouse Mutagenesis Program (Friedel et al., 2007) has succeeded in generating a murine embryonic stem cell line (EUCOMM Project ID: 24538) carrying a targeted gene-trap construct that was designed to elicit degradation of all *Dtnbp1* transcripts and to allow generation of conditional knockout mice. Characterization of these new murine alleles of the dysbindin-encoding gene would be a valuable complement to ongoing research efforts using the sandy mice.

Given that dysbindin is thought to assemble into one (or more) stable protein complex(es), characterization of mouse lines carrying null mutations in other complex subunits should provide useful insights – at the very least to test the hypothesis that the behavioural and electrophysiological phenotypes observed for sandy mice (Tables 1 and 2) reflect the lack of a biological function exerted by a protein complex of which dysbindin is part. In the case of the ternary complex proposed to contain dysbindin, WAVE2 and Abi-1 (Ito et al., 2010), these analyses would not be possible because homozygous knockout mice lacking either WAVE2 or Abi-1 die early during embryogenesis (Yamazaki et al., 2003; Ring et al., 2011). In the case of BLOC-1, several homozygous mutant lines exist in more than one genetic background. These include those carrying the 'pallid' (*Pldn*^{pa}), 'muted' (*Muted*^{mu}), 'cappuccino' (*Cno*^{cn}) and 'reduced pigmentation' (*Bloc1s3*^{rp}) alleles, all of which have arisen by spontaneous mutations. Mice homozygous for each of these mutations are viable and fertile but display pigmentation as well as platelet- and kidney-related phenotypes resembling those of homozygous sandy mice [reviewed by Li et al. (2004)]; homozygous *Bloc1s3*^{rp} mice display a relatively milder phenotype owing to partial assembly and residual activity of BLOC-1 in spite the absence of the BLOS3 subunit (Starcevic and Dell'Angelica, 2004). To our knowledge, only one study has examined these murine models of BLOC-1 deficiency for neurological abnormalities and reported motor coordination defects (Newell-Litwa et al., 2010). More detailed behavioural and electrophysiological studies on these murine lines seem warranted.

Mutant flies

Clearly recognizable orthologues of dysbindin can be found encoded by the genomes of insects such as the fruit fly, *Drosophila melanogaster*, and nematodes such as *Caenorhabditis elegans* (Cheli and Dell'Angelica, 2010). Flies homozygous for a transposon insertion within the *dysbindin* (*dysb*) gene or hemizygous for the insertion allele (over a large deletion covering the entire *dysb* gene) failed to develop the homoeostatic compensation of glutamatergic synaptic transmission that is known to occur in larval neuromuscular junctions treated with a glutamate receptor antagonist (Dickman and Davis, 2009). This phenotype was inferred to reflect a presynaptic function of dysbindin, as it could be rescued by transgenic expression of the protein in the presynaptic neuron, but not in the postsynaptic muscle cell, of neuromuscular junctions of mutant larvae (Dickman and Davis, 2009). No abnormalities in synaptic morphology were noted, and electrophysiological recordings indicated that synaptic transmission was apparently normal under baseline conditions (i.e. in the absence of the antagonist) albeit impaired when measured in the presence of low extracellular Ca^{2+} concentrations (Dickman and Davis, 2009). These observations underscore the potential of using simple model organisms (despite their large evolutionary divergence from humans) to understand the physiological roles of dysbindin.

CELLULAR MODELS OF DYSBINDIN DEFICIENCY

A number of studies have attempted to understand the cellular function(s) of dysbindin using cell lines and primary cell cultures. The published observations resulting from experiments using dysbindin-deficient primary neuronal cultures or 'neuron-related' cell lines (derived from neuroblastomas or pheochromocytomas) are summarized in Table 3. Additional published experiments have relied on uncontrolled overexpression of dysbindin (usually fused to an epitope tag) in transfected cells. Given the above-discussed evidence for association of dysbindin into one (or more) multi-subunit complex(es), interpretation of phenotypes elicited by overexpression of the dysbindin molecule alone can be problematic. In other words, would these phenotypes reflect an exacerbation of the normal function of dysbindin or a dominant-negative effect on the complex(es) containing endogenous dysbindin? Bearing this in mind, herein we restrict our discussion to reported loss-of-function phenotypes.

Two different approaches have been used to obtain dysbindin-deficient cells: (i) isolation and primary culture of cells from mutant mice, and (ii) RNAi using either siRNA (small-interfering RNA) or shRNA (short-hairpin RNA) reagents. The first approach has relied on the sandy mouse as a source of neurons (Ji et al., 2009; Kubota et al., 2009; Tang et al., 2009b), except for one study in which the pallid mouse was used as an alternative source (Ghiani et al., 2010). The sandy line has the obvious advantage of carrying a mutation in the very same gene that codes for dysbindin (Li

Table 3 **Phenotypes reported for dysbindin-deficient cell lines (neuroblastoma or pheochromocytoma) and primary neurons in culture***

Cell type	Dysbindin deficiency by	RNAi controls: Efficacy	RNAi controls: Off-target effects	Observed phenotype(s)	Reference
Immortalized cell lines					
PC12	siRNA	IB	Not shown	↑ Evoked dopamine secretion; ↑ SNAP25 protein	Kumamoto et al. (2006)
SH-SY5Y	siRNA	IB	siRNA to muted	↑ DRD2 at cell surface; ↓ CREB phosphorylation (basal and induced)	Iizuka et al. (2007)
			Not shown	↓ Neurite outgrowth; abnormal actin cytoskeleton at neurite tip; ↓ JNK phosphorylation	Kubota et al. (2009)
		IB to FLAG	Not shown	↑ MARCKS mRNA and protein	Okuda et al. (2010)
N2a	siRNA	IB	Not shown	↓ Synapsin I protein	Fei et al. (2010)
Primary neurons					
Cortical, rat	siRNA	IB	Not shown	↓ SNAP25 and synapsin I proteins; ↓ Akt phosphorylation; ↓ glutamate release; ↑ cell death upon serum withdraw	Numakawa et al. (2004)
				↑ DRD2 at cell surface; ↓ quinpirole-induced CREB phosphorylation	Iizuka et al. (2007)
Cortical, mouse	*Dtnbp1*^{sdy}	N/A	N/A	↑ DRD2 at cell surface; ↑ recycling of internalized DRD2	Ji et al. (2009)
				↑ NR2A and ↓ NR2B at cell surface	Tang et al. (2009b)
Hippocampal, mouse	*Dtnbp1*^{sdy}	N/A	N/A	↑ Exogenous NR2A-GFP at cell surface	Tang et al. (2009b)
				Abnormal cytoskeleton at growth cone; ↓ JNK phosphorylation	Kubota et al. (2009)
	Pldn^{pa}	N/A	N/A	↓ Neurite outgrowth	Ghiani et al. (2010)
Hippocampal, rat	shRNA	IB, IF	Two shRNAs; rescue	↓ Dendritic spine maturation	Ito et al. (2010)

*Cells deficient in dysbindin were obtained by RNAi or by primary culture of neurons from homozygous 'sandy' (*Dtnbp1*^{sdy}) or 'pallid' (*Pldn*^{pa}) mice, with the latter carrying a null mutation in a BLOC-1 subunit (causing secondary deficiency in dysbindin). The efficacy of RNAi was verified on the endogenous dysbindin in all cases except where 'IB to FLAG' denotes that it was tested using a transfected dysbindin-FLAG construct. DRD2, dopamine D_2 receptor; IB, immunoblotting; IF, immunofluorescence; N/A, not applicable; shRNA, short-harpin RNA; siRNA, small-interfering RNA; GFP, green fluorescent protein; CREB, cAMP-response-element-binding protein; MARCKS, myristoylated alanine-rich C-kinase substrate; SNAP25, synaptosome-associated protein of 25 kDa.

et al., 2003), thus enabling investigation of the biological function of this protein regardless of whether it acts alone or as part of any stable complex. The pallid line carries a null allele (Huang et al., 1999) in the gene encoding an essential component of BLOC-1 (Falcón-Pérez et al., 2002; Moriyama and Bonifacino, 2002), thus resulting in significantly decreased steady-state levels of other subunits, including dysbindin, and virtually no BLOC-1 activity (Li et al., 2003; Starcevic and Dell'Angelica, 2004; Ghiani et al., 2010). Using the pallid mouse as a source of neurons, the function of dysbindin as a BLOC-1 component can be investigated. The RNAi approach offers the flexibility of using immortalized cell lines or primary neurons from rats, both of which can yield more cells in culture than the primary neurons isolated from mice. However, two well-known caveats that are intrinsic to the RNAi approach need to be addressed with appropriate controls (Jackson and Linsley, 2004). The first caveat pertains to the efficacy of expression knockdown of the target gene product, as such efficacy may vary significantly depending on the specific reagent and the experimental conditions used. In most RNAi studies listed in Table 3, this caveat has been addressed by immunoblotting using antibodies against the endogenous dysbindin protein. The second caveat of the RNAi approach pertains to so-called 'off-target' effects, whereby the siRNA or shRNA reagent elicits the expression knockdown of other gene products besides the target. In contrast to some optimistic claims made by commercial vendors, off-target effects remain a widespread problem, as illustrated by a recent large-scale screening in which the effects of over 160000 constructs from three libraries (at an average of ~7 RNAi constructs per human gene) on 58 cellular phenotypes were tested (Collinet et al., 2010). Two of the RNAi studies listed in Table 3 included controls to address potential off-target effects. In one of them, Iizuka et al. (2007) reported that the phenotypes elicited in SH-SY5Y cells by treatment with a single siRNA to dysbindin could be mimicked by treatment with another siRNA designed to target the muted subunit of BLOC-1; the second siRNA can be considered an appropriate control for off-target effects of the former to the extent that the observed phenotypes are a reflection of dysbindin functioning as a component of BLOC-1. In another study, Ito et al. (2010) observed the same cellular phenotype using two independent shRNA constructs designed to target dysbindin, and in both cases the phenotype was

rescued by transient expression of an RNAi-resistant form of dysbindin.

Before discussing possible cellular functions of dysbindin in neurons and 'related' cell lines (PC12, SH-SY5Y and N2a), it is pertinent to briefly summarize the lessons learned from functional experiments using cell types such as fibroblasts and melanocytes, where the protein is thought to function exclusively as part of BLOC-1. In these cell types, the evidence implicates BLOC-1 (and, by extension, dysbindin) in protein trafficking within the endosomal–lysosomal system. In the highly pigmented human melanoma cell line, MNT-1, the dysbindin and pallidin subunits of BLOC-1 were localized by immunoelectron microscopy to transferrin-receptor-positive endosomes (Di Pietro et al., 2006), a compartment through which several integral membrane proteins travel towards melanosomes [reviewed by Raposo et al. (2007)]. BLOC-1-deficient melanocytes derived from homozygous muted mice (and shown to express reduced steady-state levels of the dysbindin and pallidin subunits) displayed gross missorting of two melanosomal membrane proteins, TYRP1 and ATP7A, which instead of being delivered to melanosomes accumulated in early endosomes (Setty et al., 2007; 2008). At least in the case of TYRP1, such endosomal accumulation in turn resulted in increased trafficking to and from the cell surface (Di Pietro et al., 2006, Setty et al., 2007) as well as to lysosomes (Di Pietro et al., 2006). BLOC-1-deficient fibroblasts (derived from the skin of pallid mice or generated by treatment with siRNA to the pallidin subunit) displayed missorting to the cell surface of at least two lysosomal membrane proteins, LAMP1 (lysosome-associated membrane protein 1; Salazar et al., 2006) and CD63 (Di Pietro et al., 2006), likely as a consequence of abnormal accumulation in early endosomes (Salazar et al., 2009). In contrast, trafficking of the transferrin receptor, which normally cycles between endosomes and the plasma membrane, was unaffected in melanocytes and fibroblasts deficient in BLOC-1 (Di Pietro et al., 2006; Setty et al., 2007). BLOC-1-deficient fibroblasts also displayed reduced steady-state levels of VAMP-7 (vesicle-associated membrane protein 7; also known as TI-VAMP) and altered distribution of syntaxin 8 (Salazar et al., 2006), with both VAMP-7 and syntaxin 8 being part of the machinery that mediates membrane fusion events involving late endosomes and lysosomes (Luzio et al., 2007).

Presynaptic functions

The first two cell-based studies on possible neuronal functions of dysbindin focused on the regulation of presynaptic proteins and vesicle release. One study reported decreased SNAP25 protein levels and glutamate release in cortical rat neurons treated with siRNA to knockdown dysbindin (Numakawa et al., 2004), whereas another reported increased SNAP25 protein levels, and normal basal secretion but increased evoked (accelerated) secretion of dopamine, in siRNA-treated PC12 cells (Kumamoto et al., 2006). The reasons for the contrasting effects observed on siRNA-mediated dysbindin knockdown in these two cell types are unclear, but it is worth noting that neither increases nor decreases in SNAP25 protein levels have been observed in hippocampus from homozygous sandy mice (Chen et al., 2008; Feng et al., 2008) or in cerebral cortex from pallid mice (Ghiani et al., 2010). On the other hand, decreased steady-state levels of the synapsin I protein, which has been implicated in synaptic transmission [albeit also in neurite outgrowth and synapse formation, reviewed by Fornasiero et al. (2010)] have been consistently observed in rat cortical neurons (Numakawa et al., 2004) and N2a cells (Fei et al., 2010) treated with siRNA to knockdown dysbindin, as well as in the cortex and hippocampal formation of homozygous sandy mice (Fei et al., 2010).

Additional evidence for presynaptic functions of dysbindin stemmed from electron microscopy analyses of asymmetrical synapses within the CA1 region of hippocampi from homozygous sandy mice, where fewer vesicles of slightly increased diameter were observed (Chen et al., 2008). Potential roles in the regulation of protein trafficking to synaptic vesicles have been investigated using brain from mice deficient in the muted subunit of BLOC-1: while partially purified synaptic vesicles contained normal levels of synaptophysin, VAMP-2, VGlut1 and VGAT, the purified fraction contained relatively higher levels of proteins normally destined for late endosomes and lysosomes, namely VAMP-7 and PI4KIIα (Newell-Litwa et al., 2009).

Postsynaptic functions

A second set of cell-based studies has focused on possible functions of dysbindin in postsynaptic terminals, in particular in the modulation of surface levels of neurotransmitter receptors. Using siRNA-treated SH-SY5Y and rat cortical neurons, Iizuka et al. (2007) observed increased surface levels of dopamine D_2 receptor (also known as DRD2). The phenotype was deemed to reflect a function of dysbindin as part of BLOC-1, as it was also observed on treating SH-SY5Y cells with siRNA to knockdown the muted subunit of the complex. In contrast to the effect observed for the D_2 receptor, the surface levels of dopamine D_1 receptor were not increased in siRNA-treated rat cortical neurons (Iizuka et al., 2007). These findings have been confirmed and expanded by two subsequent studies (Ji et al., 2009; Marley and von Zastrow, 2010), the first of them using cortical neurons from sandy mice (endogenously expressing the D_1 and D_2 receptors) and the second using siRNA-treated HEK-293 cells (stably transfected to express the receptors). Both studies demonstrated that the relative increase in D_2 receptor levels at the cell surface was not a consequence of impaired internalization but of decreased trafficking from endosomes to lysosomes and, as a secondary effect, enhanced recycling of the receptor to the plasma membrane (Ji et al., 2009; Marley and von Zastrow, 2010). Accordingly, the apparent lack of effect of dysbindin deficiency on the surface levels of the dopamine D_1 receptor would be consistent with the fact

that this receptor, unlike D2, preferentially recycles from endosomes back to the plasma membrane (for example, see Martin-Negrier et al., 2006). An analogous situation was presented when studying the effects of dysbindin deficiency (in primary neurons from sandy mice) on the trafficking of NMDA (*N*-methyl-D-aspartate) receptors containing NR2A and NR2B subunits, as those receptors containing the former preferentially traffic towards lysosomes on endocytosis and were found in higher amounts at the surface of dysbindin-deficient neurons, whereas those containing the NR2B subunit normally follow a recycling pathway and were detected in reduced amounts at the surface of mutant neurons (Tang et al., 2009b). It is worth noting that these cellular phenotypes are reminiscent of those observed for BLOC-1-deficient melanocytes and fibroblasts, in the sense that integral membrane proteins that preferentially traffic from endosomes to lysosomes (or to the lysosome-related melanosomes) are subjected to increased trafficking to the plasma membrane, due to a primary defect on the endosomes of mutant cells, while proteins that preferentially traffic from endosomes back to the plasma membrane are relatively less affected in mutant cells.

Neuronal development

Besides the presynaptic and postsynaptic abnormalities discussed in the previous sections, a few cell-based studies have documented defects in neuronal differentiation, specifically in neurite outgrowth and dendrite spine formation. Kubota et al. (2009) noticed abnormalities in the organization of the actin cytoskeleton at the tip of neurites extended by SH-SY5Y cells that had been treated first with siRNA (to knockdown dysbindin) and then with retinoic acid (to induce cellular differentiation). Similarly, the organization of the actin and tubulin cytoskeletons was somewhat abnormal in the growth cone of hippocampal neurons from sandy mice. In the case of retinoic-acid-treated SH-SY5Y cells, the extended neurites were deemed to be shorter in dysbindin-deficient cells than in control cells (Kubota et al., 2009). A partial impairment in neurite outgrowth was also observed for hippocampal neurons from pallid mice (shown to express reduced steady-state levels of the dysbindin protein), and validated by morphometric analyses that revealed reductions in not only length but also neurite number per cell (Ghiani et al., 2010). In another study, rat hippocampal neurons that had been kept in culture for several days before transfection with shRNA (to knockdown dysbindin) displayed an increased fraction of immature dendritic spines (thin spines and filopodia-like protrusions) at the expense of the fraction of mature spines (Ito et al., 2010). Whether or not these phenotypes are reflections of a common molecular mechanism of dysbindin action remains to be determined. Hypothesized molecular mechanisms include: the regulation of the actin cytoskeleton through the phosphorylation status of JNK (c-Jun N-terminal kinase; Kubota et al., 2009) or physical interaction with WAVE2 and Abi-1 (Ito et al., 2010), and the regulation of membrane delivery from endosomes to sites of plasma membrane expansion through physical interaction (in the context of BLOC-1) with SNAP25 and syntaxin 13 (Ghiani et al., 2010). In addition, the observations of reduced steady-state protein levels of synapsin I (Numakawa et al., 2004; Fei et al., 2010) and VAMP-7 (Salazar et al., 2006; Newell-Litwa et al., 2010) in cells and brains deficient in dysbindin (or in other BLOC-1 subunits) may be relevant as well, given that both VAMP-7 and synapsin I have been implicated in neuronal development and neurite outgrowth (Luzio et al., 2007; Fornasiero et al., 2010). Regardless of the exact molecular mechanism(s) underlying these phenotypes, a role for dysbindin in neuronal development would be consistent with the recent observation of a slight defect in neuronal differentiation in the dentate gyrus of sandy mice (Nihonmatsu-Kikuchi et al., 2011) and the finding that the protein is expressed in the rodent brain at much higher levels during embryonic and perinatal periods than during young adulthood (Ghiani et al., 2010; Ito et al., 2010).

CONCLUDING REMARKS

Research efforts during the last 10 years have yielded a number of unexpected and exciting findings regarding the molecular and functional properties of dysbindin. Although it is tempting to claim that its physiological significance in the central nervous system, as well as the biological plausibility of *DTNBP1* as a schizophrenia risk gene, may have been revealed, clearly much more experimental work will be necessary before a compelling case can be made. The evidence gathered so far should not be considered satisfactory as it leads to multiple alternative models of how dysbindin may act in the cell. Or are we willing to accept, based on the results currently available, that dysbindin would enter the nucleus to regulate transcription and interact with a complex involved in DNA-double-strand-break repair, and in the cytoplasm would assemble into various multi-subunit protein complexes (e.g. BLOC-1, a tripartite complex with WAVE2 and Abi-1) to regulate the cytoskeleton as well as trafficking and signalling pathways, such that multiple biologically active forms of the protein would control neurite outgrowth and dendritic spine maturation during neuronal differentiation and, in mature neurons, the biogenesis and release of synaptic vesicles at presynaptic terminals as well as the down-regulation of neurotransmitter receptors at postsynaptic terminals? A challenge for the next years will be to put these ideas, and any new ideas that might arise, to rigorous testing using a wide variety of experimental models and methodologies. Considering the tremendous progress achieved during the last 10 years, one can only be confident that our understanding of the role(s) of this protein in health and disease will be significantly improved in the near future.

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