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Gene Dosage in the Dysbindin Schizophrenia Susceptibility Network Differentially Affect Synaptic Function and Plasticity

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Neurodevelopmental disorders arise from single or multiple gene defects. However, the way multiple loci interact to modify phenotypic outcomes remains poorly understood. Here, we studied phenotypes associated with mutations in the schizophrenia susceptibility gene dysbindin (dysb), in isolation or in combination with null alleles in the dysb network component Blos1. In humans, the Blos1 ortholog Blos1s1 encodes a polypeptide that assembles, with dysbindin, into the octameric BLOC-1 complex. We biochemically confirmed BLOC-1 presence in Drosophila neurons, and measured synaptic output and complex adaptive behavior in response to BLOC-1 perturbation. Homozygous loss-of-function alleles of dysb, Blos1, or compound heterozygotes of these alleles impaired neurotransmitter release, synapse morphology, and homeostatic plasticity at the larval neuromuscular junction, and impaired olfactory habituation. This multiparameter assessment indicated that phenotypes were differentially sensitive to genetic dosages of loss-of-function BLOC-1 alleles. Our findings suggest that modification of a second genetic locus in a defined neurodevelopmental regulatory network does not follow a strict additive genetic inheritance, but rather, precise stoichiometry within the network determines phenotypic outcomes.

Key words: BLOC-1; dysbindin; olfactory habituation; schizophrenia; synaptic vesicle

Introduction
Multiple gene products converge into molecular and functional networks to influence neuronal traits, ranging from simple synapse mechanisms to complex behaviors (Kendler and Greenspan, 2006). These networks have been studied through single gene disruptions, which provide fundamental insight into the necessity and sufficiency of a single gene product for a neuronal phenotype. However, these studies assume that gene products organize into linear pathways that remain stable following disruption of a single gene, rather than dynamic networks (Greenspan, 2009). This approach is at odds with the genetics of neurodevelopmental disorders, where complex phenotypes emerge from the simultaneous modification of several genes (Stefansson et al., 2009, 2014; Bassett et al., 2010; Malhotra and Sebat, 2012; Rapoport et al., 2012; Moreno-De-Luca et al., 2013). This raises the question of how two or more genes interact to specify neuronal traits.

Here we model a two-loci genetic deficiency affecting a schizophrenia susceptibility network centered on the human gene DTNBP1 and its Drosophila ortholog dysb, both of which encode Dysbindin. We previously defined the human Dysbindin interactome (Gokhale et al., 2012), the core of which is comprised of Dysbindin and seven closely associated proteins that form an octameric complex known as the biogenesis of lysosome-related organelles complex 1 (BLOC-1; Ghiani and Dell’Angelica, 2011; Gokhale et al., 2012; Mullin et al., 2013). Polymorphisms in DTNB1 are risk factors for schizophrenia (Gornick et al., 2005; Talbot et al., 2009, 2011; Fatjó-Vilas et al., 2011; Mullin et al., 2011) and influence cognitive and neuroanatomical traits (Mullin et al., 2011; Ghiani and Dell’Angelica, 2011; Papaleo et al., 2014). Postmortem analysis revealed that 80% of schizophrenia brains contain reduced dysbindin (Talbot et al., 2004). The association of Dysbindin with schizophrenia and its inclusion into the biochemically defined BLOC-1 network suggest that combined loss-of-function mutations affecting dysbindin and the BLOC-1 network should generate predictable synaptic and circuit phenotypes, reminiscent of those in copy number variations associated with neurodevelopmental disorders.
We used *Drosophila* to understand the impact of fly loss-of-function mutations affecting BLOC-1 orthologous subunits on synaptic networks (Cheli et al., 2010). We predicted that phenotypes associated with gene copy reductions affecting BLOC-1 subunits should follow a recessive inheritance pattern, as has previously been described for BLOC-1 (Cheli et al., 2010), and that this pattern should be congruent across synaptic mechanisms that progressively scale up in complexity. We tested neurotransmitter release, synapse morphology, homeostatic plasticity, and behavioral/olfactory habituation in four BLOC-1 loss-of-function genotypes. Contrary to our prediction, homozygous loss-of-function alleles of BLOC-1 subunit genes *dysh* or *Blos1*, or compound heterozygotes in the two genes, affected synaptic functions with divergent inheritance patterns rather than the predicted simple recessive pattern. We conclude that single synapse and circuit-based phenotypes associated with a single gene-dosage imbalance are noncongruently modified by a second locus encoding an interactome component. We propose that genetic control of the stoichiometry of a neurodevelopmental regulatory network diversifies phenotypic output in normal and disease states.

**Materials and Methods**

*Drosophila* stocks, rearing, genetics, and electrophysiology. All fly stocks were reared and maintained at 25°C on normal media. For crosses, standard second and third chromosome balancers were used, *dysh*, UAS-*Dysh*, UAS-Venus-dyshbindin, *dysh*1, and *dysh*27 were obtained from Graeme Davis (University of California, San Francisco); *blos1*1118 and UAS-blos1 were obtained from Esteban Dell’Angelica (University of California, Los Angeles; Cheli et al., 2010). w1118, Canton S, Elav-GAL41555, and other fly strains such as balancer chromosome-containing and tissue-specific Elav-GAL4 driver stocks are part of the Sanyal or Ramaswami laboratory collection. *Blos1*1118 was obtained from Bloomington Drosophila Stock Center and UAS-dysh RNAi was from National Institute of Genetics.

For all physiological intracellular recordings, data were obtained from muscle 6 of abdominal segment 2 or 3 of female, wandering third-instar larvae. Recordings were only used if the resting membrane potential was between −60 and −90 mV and the muscle input resistance was >5 MΩ. For miniature excitatory junctional potential (mEJP) analysis and phantothoxin experiments, intracellular recordings were performed in modified HL3 saline containing the following (in mM): 70 NaCl, 5 KCl, 0.3 CaCl2, 1.0 MgCl2, 10 NaHCO3, 115 sucrose, 5 trehalose, and 5 BES, pH 7.2. Severed motor neurons were taken up into a stimulating electrode and stimulated at 1 Hz for 50 s. For acute pharmacological homeostasis experiments, larvae were fixed with 2% glutaraldehyde in 0.12 M Na-cacodylate buffer, pH 7.4, and postfixed with 1% osmium tetroxide in 0.12 M Na-cacodylate. Larvae were en bloc stained with 1% aqueous uranyl acetate for thin sectioning and imaging for synaptic vesicle morphology as described previously (Heerssen et al., 2008). Vesicle sizes were assessed by glycerol velocity sedimentation of high-speed head extract supernatants as described previously (van de Goor et al., 1995). Heads were collected from adult males and females as described below. Vesicle-containing fractions were detected with antibodies against cap obtained from The Developmental Studies Hybridoma Bank at University of Iowa.

**Immunoprecipitation and MS.** To determine interactions between *Drosophila* BLOC-1 subunits, fly heads from adult males and females were prepared as previously described (Roos and Kelly, 1998). Briefly, for each genotype, ~100 animals were flash frozen in liquid nitrogen and heads were separated then collected by passing the frozen tissue through a microsieve in liquid nitrogen. The frozen tissue was ground into a powder using a mortar and pestle, and combined with 100 μl lysis (Buffer A: 150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM MgCl2, pH 7.4 + 0.5% Triton X-100) with Complete anti-protease (catalog #11245200; Roche), and also frozen and ground into a powder and stored at −80°C. Samples were thawed and sonicated, tissue debris was removed by centrifugation, and protein concentration determined by Bradford assay (Bio-Rad). Proteins were resolved by SDS-PAGE on a 4−20% gel (Invitrogen) and immunoblot analysis was performed as previously described (Gokhale et al., 2012; Ryder et al., 2013). Dyna magnetic beads (catalog #655088; Dynal) were washed with 75°C sample buffer and incubated for 2 h at 4°C with 500 μg of protein extract. The beads were then washed four to six times with Buffer A (0.1% Triton X-100). Proteins were eluted with Laemmli sample buffer at 75°C. Samples were either resolved by SDS-PAGE and contents analyzed by immunoblot, or processed for MS protein identification by MS Bioworks as described previously (Gokhale et al., 2012; Ryder et al., 2013).

**Short-term olfactory habituation.** Short-term olfactory habituation was tested using a Y-maze apparatus as previously described (Das et al., 2011; Sadanandappa et al., 2013). Briefly, 4-d-old adult male and female flies were starved overnight and the naive response to ethyl butyrate (EB; 10−2 dilution in water) was tested (pre-test response). To induce short-term habituation (STH), flies were then transferred to a 125 ml glass bottle containing a suspended, 1.5 mlEppendorf with 5% EB in paraffin oil with the lid perforated for 30 min. Animals were then tested for response following the 30 min period (post-test response). To condition dysbindin knockdown experiments parenteral flies were reared at 18°C till eclosion. Newly eclosed flies (0−12 h) were shifted to 29°C, whereas the control flies were maintained at 18°C. After 4 d, flies were subjected to STH protocol, as described above—EJP monochronal (Life sciences Molecular Probes, catalog #A11120) and incubated for 2 h at 4°C with 500 μg of protein extract. The beads were then washed four to six times with Buffer A (0.1% Triton X-100). Proteins were eluted with Laemmli sample buffer at 75°C. Samples were either resolved by SDS-PAGE and contents analyzed by immunoblot, or processed for MS protein identification by MS Bioworks as described previously (Gokhale et al., 2012; Ryder et al., 2013).

**Statistical and cluster analysis.** Experimental conditions were compared using Synergy KaleidaGraph, version 4.1.3 or StatPlus Mac Built.
Results

BLOC-1 assembles into an octameric complex in Drosophila neurons

Dysbindin exists as a member of the octameric BLOC-1 in mammalian cells (Fig. 1A). While all eight mammalian BLOC-1 subunits have Drosophila orthologs, the existence of this complex in Drosophila has not yet been established, but has been predicted from yeast two-hybrid and proteomic studies (Cheli et al., 2010; Guruharsha et al., 2011). However, these studies only document a subset of the predicted interactions among the eight Drosophila BLOC-1 subunit orthologs (Fig. 1B). Thus, we first set out to determine whether an orthologous Dysbindin-containing BLOC-1 complex assembles in the Drosophila CNS neurons (Fig. 1C). We focused on this cell type based on previous work documenting the localization and requirement of Dysbindin to the presynaptic compartment (Dickman and Davis, 2009). To do this, we used Dysbindin as bait to immunoprecipitate Dysbindin and associ-
ated proteins from fly head lysates. UAS-Venus-tagged Dysbindin transgene was expressed under the pan-neuronal C155-GAL4 driver to identify neuronal proteins coprecipitating with recombinant Dysbindin in Drosophila. We prepared detergent-soluble tissue homogenates from fly heads carrying either the C155 driver alone as controls, or in combination with UAS-Venus-Dysbindin transgene to express tagged dysbindin (Fig. 1D, compare lanes 1 and 2). Venus-tagged Dysbindin from head extracts was immunoprecipitated using a GFP antibody, and immune complexes were profiled by quantitative mass spectrometry. Immunoprecipitation of Venus-Dysbindin enriched all eight BLOC-1 Drosophila orthologs compared with the C155 control, determined by spectral count quantitation (Fig. 1E). The low abundance of these proteins precluded an estimate of stoichiometry based on spectral counts. Proteins nonselectively bound to GFP beads, such as tubulin and elongation factor 1α, were similarly represented in spectral counts in both control and Venus-Dysbindin precipitated samples (Fig. 1E). The identity of the Drosophila BLOC-1 subunit orthologs was confirmed by MS/MS peptide sequencing (Fig. 1F). Due to the limited availability of Drosophila BLOC-1 antibodies, we verified the Pallidin-Dysbindin interaction by immunoprecipitation of Venus-Dysbindin and specific detection of coprecipitating Pallidin by Western blot (Fig. 1G, compare lanes 4 and 5), or by specific detection of Venus-Dysbindin in Pallidin precipitated immune complexes from Drosophila head extracts (Fig. 1H, compare lanes 3 and 5). Our data demonstrate that Dysbindin pulls down all BLOC-1 subunits in Drosophila neurons, and suggest that the architecture of Drosophila BLOC-1 highly resembles that found in the human BLOC-1 complex (Fig. 1C).

**BLOC-1 acts presynaptically to regulate quantal content at the neuromuscular junction**

The close biochemical identity of the Drosophila and mammalian BLOC-1 complexes suggests that mutations in Drosophila BLOC-1 subunits should produce recessive, gene dosage-dependent phenotypes consistent with the recessive nature of individual BLOC-1 subunit mutations in mammalian cells (Huang et al., 1999; Zhang et al., 2002; Cicciotte et al., 2003; Li et al., 2003; Gwynn et al., 2004; Starcevic and Dall’Anglicia, 2004). We analyzed whether gene-dosage reductions in BLOC-1 subunits had the capacity to produce recessive synaptic phenotypes. We used a multiparameter assessment of synaptic functions to progressively test increasing levels of synapse organization, ranging from spontaneous neurotransmitter release to circuit-based learning behavior. We began by measuring the spontaneous and evoked neurotransmitter release at the Drosophila third-instar larval neuromuscular junction (NMJ) synapse in gene-dosage imbalances affecting the BLOC-1 complex subunits Dysbindin and Blos1. The NMJ synapse is sensitive to loss-of-function alleles affecting Drosophila BLOC-1 subunits orthologs (Dickman and Davis, 2009; Dickman et al., 2012). We assessed animals for baseline mEJP amplitude and frequency, evoked EJP amplitude, and quantal content. There were no significant differences in the EJP amplitude across diverse modifications in the gene dosage of dysb, Blos1, or combinations thereof (Fig. 2F, representative traces shown in A–E). Similarly, the different BLOC-1 loss-of-function alleles had no effect on the frequency of mEJPs (Fig. 2F). Consistent with previous reports by Dickman and Davis (2009) and Dickman et al. (2012), the amplitude of the mEJP was not altered in dysb1 dysbindin mutant synapses compared with two control strains, Canton-S or w1118 (Fig. 2G). In contrast, we found that animals carrying any of three Blos1-null alleles—Blos1EV (Fig 2A, insert), Blos1cut2, and Blos1cut5—had increased mEJP amplitudes and correspondingly lower quantal content compared with both wild-type strains (Fig. 2H, L, P, respectively, red traces). While this increase in mEJP amplitude is in agreement with the initial characterization of Blos1cut2, we observed that this phenotype was consistent across all three null alleles (Cheli et al., 2010). Additionally, we found this phenotype to be dominant, as it also was present in single-copy loss of Blos1-null alleles (Fig. 2H, L, P, respectively, blue traces). Presynaptic expression of Blos1 (elav1235-Gal4; UAS-Blos1) was sufficient to rescue the observed changes in mEJP amplitude and quantal content (Fig. 2C, K, O). These data demonstrate that Blos1 presynaptically regulates mEJP amplitude and that this phenotype is a dominant rather than a recessive trait of Blos1-null alleles.

The distinct effect of the Blos1 alleles and lack of effect of the dysbindin alleles on mini amplitude suggested the following two possibilities. First, Blos1 and dysb participate in different molecular and genetic networks, a hypothesis seemingly at odds with Blos1 and Dysbindin being subunits of the BLOC-1 complex. Second, dysb alleles reduce BLOC-1 function to a different extent compared with Blos1-null mutants, suggesting a BLOC-1 complex loss-of-function threshold under which the mEJP phenotype is expressed. To discriminate between these possibilities, we tested the ability of dysb hypomorphic mutants (dysb1) and the deletion Df (BSC 416), which entirely deletes the dysb gene as well as ~20 flanking genes (Dickman and Davis, 2009) to genetically interact with Blos1-null alleles in regulating mEJP amplitude and quantal content at the NMJ. Interestingly, we found that the dysb1 allele expressed in trans with a single-copy loss of Blos1 rescued the mEJP amplitude and quantal content back to wild-type levels (Fig. 2I, M, Q, compare red and blue traces). We observed the same result in flies double heterozygous for Df (BSC 416) and the Blos1F3-null mutation (Fig. 2J, compare red and blue traces). As expected, Blos1 expressed in trans with the dysb1EV, which is a perfect excision of the dysb1 mutation, behaved similar to a single-copy loss of Blos1 (Fig. 2N, compare red and blue traces). Further, we found that the ability of dysb alleles to modify Blos1 mEJP and resultant quantal content phenotypes is presynaptic, as adding back Dysbindin presynaptically (elav1235, Gal4; UAS-dysb) in the Blos1EV; dysb1/1 transheterozygous animal did, in fact, lead to increased mEJP amplitude and reduced quantal content, similar to the effect observed in single-copy loss of Blos1 (Fig. 2R, compare red and green traces).

The increased mEJP amplitude observed in Blos1-null alleles could result from an increase in synaptic vesicle size. Therefore, we determined synaptic vesicle sizes using glycerol velocity sedimentation of adult head high-speed supernatants (Fig. 3A) as well as electron microscopy of the larval NMJ (Fig. 3B–F). We found no differences in synaptic vesicle sizes in Blos1-null animals compared with controls (Fig. 3A, B) or Blos1EV; dysb1/1 animals (Fig. 3A) that display normal amplitude mEJPs. From this, we conclude that the mEJP data demonstrate that BLOC-1 subunits participate in a common presynaptic molecular and genetic network to regulate baseline neurotransmission at the Drosophila NMJ in a manner independent from vesicle size. Additionally, these data show that neurotransmission is sensitive to the gene dosage and precise stoichiometry of BLOC-1 subunits Dysbindin and Blos1 rather than an additive genetic effect of sequential reduction of individual subunits predicted from a recessive trait.
Normal synaptic growth and morphology require BLOC-1 function

Spontaneous neurotransmission regulates synapse function and morphology, and is required in *Drosophila* for synaptic structural maturation (Sutton et al., 2004; Choi et al., 2014). For example, gene mutations associated with neurodevelopmental disorders, such as in dFMRP, affect the morphology of *Drosophila* synapses while displaying spontaneous release phenotypes reminiscent of the *Blos1*-null phenotype (Reeve et al., 2005; Gatto and Broadie, 2008; Zhao et al., 2013). Therefore, we explored the morphology of the neuromuscular junction in BLOC-1 loss-of-function allele combinations. We sought to test whether synapse morphology phenotypes followed the dominant inheritance defined by the spontaneous release. We performed anti-HRP immunohistochemistry of larval muscle 6/7 synapses to quantify the number of boutons per unit muscle area (Fig. 4). We determined that *Blos1*-null alleles have significantly increased number of boutons per muscle at the 6/7 synapse (Fig. 4J, compare A with B, C). This increase in bouton number occurred without changes in muscle size across genotypes (Fig. 4K). The increased number of boutons was a dominant phenotype as it was also observed in heterozygous *Blos1* mutants (Fig. 4J, compare A with D, E), a
Figure 3. Blos1-null alleles possess normal-sized synaptic vesicles and presynapse ultrastructure. A, Adult head homogenates were differentially centrifuged and high-speed supernatants resolved by glycerol velocity sedimentation. Synaptic vesicles in fractions were detected by csp immunoblotting (IB). Csp-positive vesicles peaked at the same fractions regardless of the BLOC-1 genotype. B, Representative transmission electron micrographs of larval wild-type (W1118) and Blos1-null (Blos1<sup>ex2</sup>) NMJs. Inserts correspond to threefold magnification of areas boxed. C, D, F, Dot plots of synaptic vesicle density, diameter, and the number of T-bars per presynapse, respectively. E, frequency plot of synaptic vesicle diameters. No differences were detected among genotypes. All comparisons were made with Wilcoxon–Mann–Whitney test. NS indicates nonsignificant p values. Dot plots depict number of animals analyzed. Black represents wild-type and blue symbols Blos1-null animals, respectively.

dysbindin and Blos1 are necessary for the function of synaptic vesicle pools

BLOC-1 loss-of-function allelic combinations have a one-to-one correspondence between genotypes and the bouton morphology and spontaneous release phenotypes. Thus we asked whether this genotype-to-phenotype congruence was observed across multiple synaptic organization levels. We hypothesized that increasingly complex synaptic functions would be similarly sensitive to reductions in the genetic dosage of neuronal BLOC-1 subunits if a common BLOC-1-dependent molecular mechanism controls these synaptic functions. In contrast, if synaptic processes were to respond divergently to the same genetic dosage imbalances, it would indicate that different BLOC-1-dependent molecular mechanisms are required for diverse synapse functions. To differentiate between these hypotheses, we assessed the effect of BLOC-1 genetic dosage on the mobilization of synaptic vesicle pools in response to either a philanthotoxin or high-frequency stimulation challenge (Delgado et al., 2000; Dickman and Davis, 2009; Frank et al., 2013). We focused on the Blos1<sup>EY</sup>;dysb<sup>i</sup> transheterozygote, as the ability for the dysb<sup>i</sup> mutations to preclude the dominant effects of Blos1 on mEJP amplitude suggests that synaptic mechanisms require precise BLOC-1 subunit stoichiometry rather than an additive reduction of BLOC-1 complex function.

We first tested the effects of BLOC-1 loss-of-function alleles on philanthotoxin-induced presynaptic homeostatic compensation at the NMJ. In response to an acute, 10 min incubation with philanthotoxin, which irreversibly blocks non-NMDA glutamate receptors, wild-type animals exhibit robust homeostatic compensation to this postsynaptic block, as observed through a reduction in mEJP amplitude while maintaining the amplitude of their EJP (Fig. 5A–A1, compare black and blue traces; Dickman and Davis, 2009; Dickman et al., 2012; Davis, 2013; Frank et al., 2013; Frank, 2014). This synaptic compensation occurs by doubling their quantal content of neurotransmitter release over baseline (Fig. 5H). However, dysb<sup>i</sup> mutants failed to display such presynaptic homeostatic compensation in response to this toxin, as reflected by the unchanged quantal content after toxin incubation (Fig. 5B, H, compare black and blue traces). Importantly, single-copy loss of dysbindin does not block synaptic homeostasis (data not shown). Unlike what we observed with the mEJP and branching dominant phenotypes, Blos1-null (Fig. 5C, H) and
heterozygous animals (Fig. 5D, H) have no defect in synaptic compensation. However, transheterozygotic Blos1EY;dysb1 mutants blocked synaptic homeostasis to the same extent as dysb1 homozygotic mutants (Fig. 5A, H, compare B, E). These results demonstrate that the BLOC-1 complex is necessary for homeostatic synaptic plasticity, yet the BLOC-1-dependent molecular mechanisms that synaptic homeostasis relies on differ from those required by spontaneous release and synapse morphology. This incongruence in inheritance supports the hypothesis that synaptic mechanisms are differentially sensitive to BLOC-1 gene-dosage imbalances, which do not follow a simple recessive reduction of BLOC-1 complex function.

Increased quantal content during presynaptic homeostatic compensation requires a calcium-dependent increase in size of the readily releasable pool. Thus, we hypothesized that BLOC-1 loss-of-function allele combinations could alter the mobilization of synaptic vesicle pools following the genotype-to-phenotype pattern observed in homeostatic plasticity. To test this hypothesis, we measured the size of the total vesicle pool and the recycling pool in different BLOC-1 mutants. To do this, NMJ responses were recorded under different stimulation frequencies in the presence of the vATPase inhibitor bafilomycin A1. This inhibitor prevents reloading of neurotransmitter into synaptic vesicles after exocytosis. Low-frequency stimulation (3 Hz; Fig. 6A–C), engages the recycling pool of vesicles to maintain neurotransmission; however, intense, high-frequency stimulation (10 Hz; Fig. 6D, E) mobilizes the reserve pool of vesicles to support neurotransmission (Delgado et al., 2000; Kim et al., 2009). None of the loss-of-function allele combinations affected the rate of vesicle depletion at low-frequency stimulation (Fig. 6A–C), indicating that BLOC-1 is not required for the mobilization or engagement of the recycling pool. However, we found that Blos1 homozygous mutants (Fig. 6E) but not dysb1 mutants (Fig. 6D) had a significantly enhanced depletion rate of the total vesicle pool compared with wild-type animals and Blos1 single-copy loss. The divergent effects of the Blos1 and dysb1 homozygous mutants on the mobilization of the total vesicle pool did not reflect BLOC-1 independent functions of these alleles as evidenced by transheterozygotic Blos1EY;dysb1 mutants, which phenocopied the Blos1 homozygous animals when stimulated at 10 Hz (Fig. 6F). The accelerated rate of depletion of the total vesicle pool paired with a lack of effect on the recycling vesicle pool indicates a selective perturbation to the reserve pool of vesicles in these animals. We conclude that the reserve pool necessitates the BLOC-1 complex, but this BLOC-1 requirement molecularly differs from the BLOC-1 requirement for synaptic homeostatic plasticity.
Dysbindin and Blos1 are required for olfactory short-term habituation in Drosophila

The divergent effects of BLOC-1 subunit gene-dosage reductions among four NMJ synapse phenotypes prompted us to ask how would a learning behavior respond to BLOC-1 subunit gene-dosage reductions? To address this question, we assessed BLOC-1 mutants in a short-term olfactory habituation assay. Short-term olfactory habituation in adults involves a retrograde signal and mobilization of the reserve pool of vesicles to potentiate GABAergic neurotransmission in the local interneurons (LNs) of the olfactory circuit in Drosophila (Das et al., 2011; Sadanandappa et al., 2013; Twick et al., 2014). Thus, we predicted that this complex learning behavior in adult flies would be affected by BLOC-1 mutations following a genotype-to-phenotype pattern similar to the reserve pool phenotypes (Fig. 6). We explored genotype-to-phenotype relationships across different combinations of BLOC-1 loss-of-function alleles (Fig. 7). BLOC-1 mutant flies are healthy and viable, with no defects in locomotion, and can therefore be tested in behavioral assays (data not shown). We trained wild-type and mutant animals with a 30 min aversive odor exposure (5% ethyl butyrate) to determine odorant-specific reductions in avoidance behavior, indicating a deficit in short-term olfactory habituation (Fig. 7A). While the hypomorph dysb¹ animals are viable and healthy, the BSC 416 dysb deficiency flies are not. Therefore, to test if the habituation phenotype was dysb allele specific, we expressed the dysb¹ mutation in trans with the dysbDf. Single-copy loss of dysb, either by the dysb¹ or the dysbDf mutation, did not affect habituation (Fig. 7A). However, short-term olfactory habituation was impaired in dysb¹ animals in trans with the dysbindin deficiency (BSC 416; Fig. 7A) confirming that loss of dysbindin inhibits short-term olfactory habituation.

Short-term olfactory habituation requires plasticity in the GABAergic local interneurons and antennal lobe projection neurons, which form a circuit that relays information from olfactory sensory neurons to higher centers, the mushroom bodies and the lateral protocerebrum (Das et al., 2011; Sadanandappa et al., 2013; Twick et al., 2014). Thus, we predicted that this complex learning behavior in adult flies would be affected by BLOC-1 mutations following a genotype-to-phenotype pattern similar to the reserve pool phenotypes (Fig. 6). We explored genotype-to-phenotype relationships across different combinations of BLOC-1 loss-of-function alleles (Fig. 7). BLOC-1 mutant flies are healthy and viable, with no defects in locomotion, and can therefore be tested in behavioral assays (data not shown). We trained wild-type and mutant animals with a 30 min aversive odor exposure (5% ethyl butyrate) to determine odorant-specific reductions in avoidance to a second odor exposure.

Wild-type flies exhibit olfactory habituation as determined by a reduced avoidance response by prior exposure to ethyl butyrate (Fig. 7A, CS, compare pre- and post-test in blue). In contrast, animals homozygous for the dysb¹ mutation showed no reduction in avoidance behavior, indicating a deficit in short-term olfactory habituation (Fig. 7A). While the hypomorph dysb¹ animals are viable and healthy, the BSC 416 dysb deficiency flies are not. Therefore, to test if the habituation phenotype was dysb allele specific, we expressed the dysb¹ mutation in trans with the dysbDf. Single-copy loss of dysb, either by the dysb¹ or the dysbDf mutation, did not affect habituation (Fig. 7A). However, short-term olfactory habituation was impaired in dysb¹ animals in trans with the dysbindin deficiency (BSC 416; Fig. 7A) confirming that loss of dysbindin inhibits short-term olfactory habituation.

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Dysbindin knockdown to the adult circuit using the transcriptional repressor tub-Gal80ts (Fig. 7C, 18°C). Repression of Gal80ts at the nonpermissive temperature led to the expression of dysbindin-RNAi and impaired olfactory habituation phenotype (Fig. 7C, 29°C), whereas at the permissive temperature of 18°C, olfactory habituation was normal. These data demonstrate that Dysbindin is acutely necessary in the local interneurons and projection neurons during adult Drosophila olfactory habituation.

We next asked whether the observed defect in short-term habituation was specific to loss of the Dysbindin subunit, or if it could be attributed to the BLOC-1 complex. Homozygous loss of Blos1 prevented olfactory short-term habituation (Fig. 7D). Similar to dysbindin, however, a single-copy loss of Blos1 was insufficient to produce the phenotype. While a single-copy loss of each subunit did not preclude short-term habituation, transheterozygotic expression of these mutations did prevent olfactory short-term habituation (Fig. 7E, F). We conclude that BLOC-1 is required for olfactory short-term habituation in Drosophila. These results indicate that the genotype-to-phenotype relationships observed for the reserve vesicle pool differ from those observed in olfactory short-term habituation as illustrated by the dysb homozygous loss-of-function alleles. These data show that the BLOC-1 complex requirements follow different genotype-to-phenotype relationships.

Hierarchical clustering analysis of BLOC-1 genotype and associated phenotype

We used the array of genotypes and phenotypes exploring BLOC-1 synaptic functions to quantitatively determine cosegregation of traits with genotypes. We analyzed genotype-to-phenotype pairs using single linkage hierarchical clustering (Fig. 8). We assigned a value of 0 to wild-type and 1 to mutant phenotypes, respectively. Clustering revealed that a homozygous dysb null genotype (Fig. 8A, Blos1/dysb) is phenotypically closer to a Blos1 heterozygote (Fig. 8A, Blos1/H11002/H11002) than to homozygous dysb loss-of-function genotype (Fig. 8A, dysb/H11002/H11002). In contrast, the phenotypes observed in homozygous dysb loss-of-function-null flies (Fig. 8A, dysb/H11002/H11002) cosegregated better with a dysb, Blos1 transheterozygote (Fig. 8A, Blos1/H11002/dysb). Similarly, mEJP amplitude and branching dominant traits were clustered together and away from the presynaptic homeostasis, reserve pool, and habituation phenotypes. Phenotype and genotype clustering were different from randomized phenotype-genotype pairs (Fig. 8B). This analysis shows that an array of six genotypes and five traits identify as a minimum two nonoverlapping phenotypic clusters within a collection of BLOC-1 loss-of-function mutations. Our findings demonstrate that alleles reducing the function of the BLOC-1 protein complex at the synapse are modified by a second complex subunit-encoding locus. However, these genetic interactions depart from Mendelian inheritance even though alleles affect the same restricted molecular network.
**Figure 7.** BLOC-1 is required at LNs and projection neurons for short-term olfactory habituation in Drosophila. Following aversive odor exposure, control (Canton S, CS) or single-copy loss-of-function (dysb 1/+, dysb 1/1) animals show significant avoidance behavior, represented as % Response, compared with pre-exposure response (A, compare blue dots to black). Dysb mutants (dysb 1 and dysb 1/Df) show no learned avoidance following aversive odor exposure. B, Specific expression of dysbindin RNAi in either the LNs (LN1 driver) or projection neurons (GH146 driver) is sufficient to prevent normal postexposure avoidance response. C, Inhibition of dysbindin RNAi expression in the LNs or the projection neurons using the temperature-sensitive repressor tubGal80ts at the permissive temperature (18°C) allowed for normal olfactory habituation. Repression of tubGal80ts at the nonpermissive temperature (29°C) led to impaired odorant-avoidance response. D, Blo1 homozygous mutations but no single-copy loss prevent learned odorant-avoidance behavior. E, Blo1 mutations expressed in trans with dysb 1 prevent learned odorant-avoidance response. Dot plots represent independent experiment per genotype. Comparisons were made with Wilcoxon–Mann–Whitney test.

**Figure 8.** Hierarchical clustering analysis of BLOC-1 genotype and their associated phenotypes. Genotype-to-phenotype pairs were analyzed by single linkage hierarchical clustering. Phenotypes are assigned a value of 0 to wild-type (blue) and 1 to mutant phenotypes (orange), respectively. A, Depicts experimentally generated genotype-to-phenotype pairs. B, Represents randomly assigned genotype-to-phenotype pairs. Hierarchical clustering analysis was performed for A and B and cluster r values are presented in A: r experimental (r_exp) and r random (r_ran). All experimentally defined cluster r correlations are different from those predicted from random genotype-to-phenotype pairs. See text for details.
Discussion

Here, we examined the impact of combined loss-of-function mutations affecting the BLOC-1 complex on synaptic neurotransmission in Drosophila. BLOC-1 is a closely associating octameric protein complex whose interaction network we defined in the fly through the schizophrenia susceptibility factor and BLOC-1 subunit Dysbindin (Fig. 1; Cheli et al., 2010; Guruharsha et al., 2011). We found that homozygous loss-of-function alleles of dysb, Blos1, or compound heterozygotes of these alleles impaired diverse presynaptic mechanisms. These identified deficits affect mechanisms of increasing complexity, from abnormal spontaneous neurotransmitter release and synapse morphology at the neuromuscular junction to olfactory habituation. This multiparameter assessment indicated that phenotypes were differentially sensitive to genetic reductions of BLOC-1 function in a way that departs from the predicted recessive inheritance of dysbindin loss of function. On one extreme, spontaneous neurotransmission and synapse morphology at the NMJ follow a dominant inheritance in response to Blos1 loss of function (Figs. 2–4). This phenotype is rescued by a second loss-of-function allele in dysb. This is particularly striking when we consider that the polypeptides encoded by these genes form a complex, as we demonstrated in Drosophila neurons (Fig. 1). In contrast, short-term olfactory habituation behaves as a recessive character (Fig. 7). We draw two conclusions from these findings that we would like to focus on. First, gene-dosage reductions in two or more genetic loci encoding BLOC-1 polypeptides, which belong to the same protein interaction network, do not confer strictly additive functional consequences. Second, genotype-to-phenotype correlations observed in a BLOC-1 trait following a gene pair analysis better predict, although not precisely, how other traits may respond. These findings provide a perspective to the complexity and predictability of synaptic phenotypes derived from copy number variation associated to human neurodevelopmental disorders (Stefansson et al., 2009, 2014 Bassett et al., 2010; Malhotra and Sebat, 2012; Rapoport et al., 2012; Moreno-De-Luca et al., 2013; Ahn et al., 2014).

Dominant and recessive fly traits associated with mutations affecting BLOC-1 complexes support the dosage balance hypothesis, which predicts that mutations affecting genes encoding different subunits of a protein complex may confer distinct phenotypes and inheritance mechanisms (Veitia et al., 2008; Birchler and Veitia, 2012). The ultimate result is a range of resultant subcomplex remnants spanning from total complex depletion to combinations of residual subunits, referred to here as remnants. These remnants have been described in mice carrying mutations in genes encoding BLOC-1 complex subunits, including dysbindin. However, no phenotype has been assigned to these remnants to date (Huang et al., 1999; Zhang et al., 2002; Cicciotte et al., 2003; Li et al., 2003; Gwynn et al., 2004; Starcevic and Dell’Angelica, 2004; Yang et al., 2012). We postulate that olfactory habituation, a BLOC-1 recessive phenotype, is at one end of this spectrum and requires fully assembled octameric BLOC-1, which would be disrupted by any BLOC-1 subunit mutation. Further, olfactory habituation mechanisms would be unaffected by BLOC-1 remnants (Fig. 9). In contrast, traits that depart from a recessive inheritance pattern and display diverse responses to BLOC-1 subunit mutations may be caused by at least three nonmutually exclusive mechanisms related to the dosage balance hypothesis. First, mutations in different BLOC-1 subunits may result in similar reductions of BLOC-1 content and activity. However, traits may be differentially sensitive to total BLOC-1 cellular content. Second, divergent phenotypes in response to gene-dosage reductions may reflect different functions engaged by distinct BLOC-1 subunits, performed by either monomeric subunits or monomers as part of other protein complexes. Finally, loss-of-function mutations affecting BLOC-1 subunits may lead to gain-of-function remnants (Fig. 9). Thus, some phenotypes may emerge from these BLOC-1 complex remnants left over after uneven protein downregulation of the octamer. The remnant hypothesis best explains the mEJP amplitude phenotypes, which lie at the other end of the spectrum from olfactory habituation. If increased mEJP amplitude is caused by Blos1 mutations leaving behind “deleterious,” Dysbindin-containing subcomplex remnants, then reducing Dysbindin levels by dysb mutations should restore mEJP amplitude to wild-type levels. This prediction is satisfied by our results with multiple Blos1 and dysb allele combinations (Fig. 2). Thus, the mEJP amplitude phenotype suggests the existence of a class of neurodevelopmental disease phenotypes that do not simply result from network loss of function but rather from changes in the stoichiometry of network components. It is possible that a loss-of-function allele in DTNBP1 or other gene may increase the risk or be causative of a disease trait when in isolation yet have no consequences when combined with another gene defect affecting the network. We speculate that philanthotoxin-induced homeostasis and reserve pool mobilization are differentially sensitive to BLOC-1 rem-
nants, and that these phenotypes reside somewhere in the middle of this spectrum.

In this study, we modeled a two-gene synaptic neurodevelopmental defect in the fly in an effort to comprehend how multiple genes influence synaptic functions that may be compromised in schizophrenia. Our strategy is founded on the polygenic character of schizophrenia, illustrated by copy number variations that strongly confer disease risk (Gottesman and Shields, 1967; Mirnics et al., 2000; Purcell et al., 2009, 2014; Faludi and Mirnics, 2011). Here, we focus on the product of a gene associated to schizophrenia susceptibility and a modulator of brain structure and cognition in normal humans, dysbindin (Straub et al., 2002; Van Den Bogaert et al., 2003; Bray et al., 2005; Luciano et al., 2009; Markov et al., 2009, 2010; Mechelli et al., 2010; Cerasa et al., 2011; Mullin et al., 2011; Tognin et al., 2011; Wolf et al., 2011; Ghiani and Dell’Angelica, 2011; Ayalew et al., 2012; Papaleo et al., 2014). Moreover, similar to Drosophila dysb alleles, cognitive traits associated to alleles of the human dysb ortholog, DTNBP1, are modified by a second locus in a human dysbindin functional interactome (Papaleo et al., 2014). Finally, mutations in Drosophila genes encoding the BLOC-1 subunits Dysbindin and Blos1 preclude short-term olfactory habituation, as established here (Fig. 7). We draw several parallels between our analyses in the fly and observed deficits in schizophrenia, which make our study of particular relevance. First, Drosophila olfactory habituation is mediated by GABAergic interneurons, which modulate the output of odorant-selective projection neurons to mushroom bodies (Das et al., 2011; Sadanandappa et al., 2013; Twick et al., 2014). We demonstrate the requirement of BLOC-1 function in these interneurons in short-term olfactory habituation. GABAergic interneuron dysfunction is also observed in mice lacking dysbindin (Carlson et al., 2011; Larimore et al., 2014), as well as in both the prefrontal cortex and hippocampus of schizophrenia patients (Benes and Berretta, 2001; Beasley et al., 2002; Hashimoto et al., 2003, 2008; Nakazawa et al., 2012). Second, impaired sensory habituation is a common manifestation in schizophrenia subjects (Geyer and Braff, 1987; Braff et al., 1992; Holt et al., 2005; Williams et al., 2013; Hu et al., 2014). In humans, sensory habituation defects are considered an intermediate, or “endophenotype.” In complex genetic disorders such as schizophrenia, endophenotypes may serve as useful biological markers, bridging diagnostic phenomenology with cellular and molecular mechanisms of disease (Gottesman and Gould, 2003). As such, our studies are the first example of deconstructing an endophenotype, sensory habituation, into lower complexity synaptic mechanisms in Drosophila. We measured distinct functional properties of synapses that could be substrates of defective sensory habituation. While none of the synaptic functions assessed precisely matched their response to combinations of gene-dosage reductions, sensory habituation clustered with synaptic plasticity mechanisms observed during high-frequency stimulation and phlantotoxin-induced synaptic homeostasis (Fig. 8). Clustering of phenotypes and their underlying mechanisms is most clearly perceived through the study of combined heterozygotic gene defects. These findings demonstrate that mechanistic deconstruction of an endophenotype is better understood through assessments spanning different levels of synaptic organization and complexity, as well as through genetic perturbations of two or more genes encoding network components.

References


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