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Regulation of *Drosophila* Synaptic Function and Plasticity by a Schizophrenia Susceptibility Network.

By

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Neuroscience

2014

Abstract

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By Ariana Paone Mullin

Neurodevelopmental disorders (NDDs) are genetically complex, arising from single or multiple gene defects, and include schizophrenia, intellectual disability, and autism spectrum disorder. Many NDDs, particularly those associated with large chromosomal deletions, either share common genetic variations or it is postulated that the associated gene products converge into a common molecular or cellular pathway. However, the way multiple loci interact to modify phenotypic outcomes remains poorly understood. Additionally, current studies focus on monogenic NDDs because of their straightforward study and conceptualization, despite the involvement of multiple loci. Currently, there are no studies exploring the interactions of multiple genes or gene products associated with these disorders and their effects at the synapse. Here, I use a biochemically curated interaction network centered around the schizophrenia susceptibility gene *dysbindin (dysb),* the Drosophila ortholog of the human gene *DTNBP1.* In this study, I examined the 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 *Bloc1s1* encodes a polypeptide that assembles, with dysbindin, into the octameric BLOC-1 complex. Here, I 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. Further, I identified the N-Ethylmaleimide Sensitive Factor (NSF) as a factor sensitive to BLOC-1 deficiency. I used NSF to test the hypothesis that molecular and genetic interactors converge into a functionally-defined pathway. My 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. Additionally, I demonstrate that a biochemically curated interactome can be used to direct investigation of pathways associated to complex genetic diseases, such as schizophrenia and related neurodevelopmental disorders. Together, this work supports the investigation of neurodevelopmental disorders through the assessment of multiple endophenotypes in response to polygenic experimental manipulations to better approximate complex disease states.

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"And now, let the wild rumpus start!"

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Key Terms and Abbreviations

NDD	Neurodevelopmental Disorders
AP-3	Adaptor Protein complex- 3
GWAS	Genome Wide Association Study
BLOC-1	Biogenesis of Lysosome-related Organelles Complex-1
dysb^{1}	*Drosophila* dysbindin mutant allele; piggy-back insertion; hypomorph
dysb^{DF}	*Drosophila* dysbindin 'deficiency' allele; gene deletion
dysb^{RV}	*Drosophila* dysbindin 'revertant' allele; precise excision of dysb^{1} insertion
Blos1^{EY}	*Drosophila* blos1 null allele; p-element insertion in the fly line EY0629
Blos1^{ex2}	*Drosophila* blos1 mutant allele, deletion in blos1 Δ $^{-}$56-38
Blos1^{ex65}	*Drosophila* blos1 mutant allele, deletion in blos1 Δ $^{-}$56-1033
NMJ	Neuromuscular junction
EJP	Excitatory Junctional Potential
mEJP	miniature Excitatory Junctional Potential
RP	Reserve Pool
RRP	Readily Releasably Pool
SNARE	SNAP receptor protein
SNAP	soluble NSF attachment protein
NSF	N-Ethylmaleimide Sensitive Factor
PhTx	Philanthotoxin
STH	Short-term olfactory habituation

Homologous Human Genes and Encoded Proteins in *Drosophila*

Human Gene	Human Protein	*Drosophila* Gene	*Drosophila* Protein
FMR1	fmr1, FMRP	*dfmr1*	dFMR1
DTNBP1, BLOC1S8	Dysbindin, BLOC1s8	*dysbindin, dysb*	dysbindin, dysb
BLOC1S1, BLOS1	BLOC1s1, BLOS1	*blos1*	blos1
NSF	NSF	*comt, Nsf,dNsf1*	dNsf-1

CHAPTER I

GENERAL INTRODUCTION

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Overview and Significance

Our understanding of complex neurodevelopmental disorders, including schizophrenia, intellectual disability, and autism spectrum disorder, has greatly increased in recent decades (Doherty, O'Donovan et al. 2012, Rapoport, Giedd et al. 2012, Adam 2013). In particular, several key genetic components of inherited neurodevelopmental disorders have been identified, and the cellular role of their gene products have been the focus of many experimental investigations (Amir, Van den Veyver et al. 1999, Doherty, O'Donovan et al. 2012). However, there are several questions that remain unanswered, and many disorders for which no good experimental model exists. In particular, many neurodevelopmental disorders seem to arise from chromosomal deletion syndromes, where a single copy of numerous genes is lost (Bassett, Scherer et al. 2010, Malhotra, McCarthy et al. 2011, Malhotra and Sebat 2012). This is exemplified by the 22q11.2 deletion syndrome, which results in the loss of between 35-60 genes, and is among the highest genetic associations for risk of neurodevelopmental disorders (Murphy 2002, Ahn, Gotay et al. 2014, Schneider, Debbane et al. 2014). Classical genetic studies have attempted to understand these hemideletion syndromes through removal of a gene of interest in order to dissect the role of the associated gene product. This approach has been invaluable in advancing our understanding of certain monogenic disorders, such as in the case of identification of the *Fragile X mental retardation 1* gene, which encodes the Fragile X Mental Retardation Protein (FMRP). First identified in 1991, *FMR1* contains a trinucleotide expansion repeat, which is now known to cause complete silencing of *FMR1* and subsequent loss of FMRP (Verkerk, Pieretti et al. 1991, Oostra and Verkerk 1992,

Feng, Zhang et al. 1995). Laboratory research following this discovery led to elucidating essential roles for FMRP in proper brain development (Reiss, Abrams et al. 1995, Comery, Harris et al. 1997, Morales, Hiesinger et al. 2002). In the laboratory, however, studying the effects of the loss of a single gene and it's encoded polypeptide does not address the complicated genetics at play in large hemideletion syndromes, where loss of a single copy of multiple genes, rather than total removal of just one, gives rise to the observed phenotypes (Gottesman and Shields 1967, Purcell, Wray et al. 2009).

Despite recent advancements and identification of at risk alleles, we have yet to adequately identify disease mechanisms that will inform appropriate treatments for neurodevelopmental disorders. These gaps in our understanding suggest that our attempts to conceptualize these disorders as arising from a single genetic modification, as well as the notion that interactome components participate in a linear genetic pathway that remains stable and intact following single gene disruption, are no longer sufficient in advancing our knowledge of these disorders. Here, I attempt to address these challenges in the field and reconstruct our understanding of the genetics of complex neurodevelopmental disorders. To do this, I will consider the gene *DTNBP1*, polymorphisms of which are associated with the development of schizophrenia as well as more severe forms of the disease such as early onset childhood schizophrenia (Gornick, Addington et al. 2005, Allen, Bagade et al. 2008, Talbot, Ong et al. 2009, Schizophrenia_Research_Forum 2010, Fatjó-Vilas, Papiol et al. 2011, Mullin, Gokhale et al. 2011). This gene is ideally suited as the object of this study, as its

gene product, dysbindin, has several known interacting proteins that together form a larger protein complex (BLOC-1)(Starcevic and Dell'Angelica 2004, Ghiani, Starcevic et al. 2010), the known biology of which I will describe shortly. Additionally, this gene and it's gene product, as well as the interacting proteins, are evolutionarily conserved (Cheli, Daniels et al. 2010), allowing for the study of dysbindin and the related BLOC-1 complex across vertebrate and invertebrate experimental systems. Thus, I will exploit these characteristics of *DTNBP1* and dysbindin to address the following questions:

First, to what extent do loss-of-function mutations within an interactome or genetic pathway phenocopy each other? We would predict that perturbations to different subunits within a protein complex, such as the aforementioned BLOC-1, should result in identical molecular and functional phenotypes. That is to say that if multiple proteins equally participate in the same cellular events, loss of any one of those proteins should yield the same output across multiple assessments. Alternatively, it would also follow that combinations of loss-of-function mutations across multiple proteins within the complex should also yield identical phenotypes.

Second, if we now consider that same, single loss-of-function mutation, how does this mutation affect a molecularly defined network, and to what extent are these network components altered in response to the single loss-of-function? As described above, previous work has assumed that network components remain stable even in the face of disruption of a network component. However, I predict that this is not the case, and that loss-of-function mutations may actually lead to

network-wide instability. Additionally, I predict that components of a network that are sensitive to a loss-of-function mutation may converge in a functionally defined pathway.

In addressing these two key questions, I will demonstrate that a single genetic perturbation results in a complex set of changes in cellular activity in multiple experimental models, from human-derived cells, to synaptic activity and simple learning behavior in *Drosophila*. Additionally, I demonstrate that by using a molecularly defined network to guide investigation, we can understand how network components converge in a functionally defined pathway. This work highlights the importance of taking into account network interactions when experimentally perturbing a network constituent. Additionally, my research provides novel insight into the challenges of studying complex polygenic neurodevelopmental disorders by highlighting the range of phenotypes that can arise from combinations of loss-of-function mutations to multiple alleles, albeit within a predefined network associated with neurodevelopmental disorders rather than across a genetic loci containing multiple protein networks. The findings of this study, however, provide caveats equally to both those studying a single gene as a proxy for the behavior of the complex to which the encoded protein belongs, as well as those attempting to recapitulate a complex genetic disorder through single gene approaches.

Here, I will study the octameric BLOC-1 complex as an example of a defined protein network implicated in neurodevelopmental disease. By choosing this complex as the subject of my work, I also contribute to our understanding of the

role of BLOC-1 in stages of synaptic vesicle biogenesis and cycling to and from the plasma membrane. Additionally, I describe a previously uncharacterized role for BLOC-1 in maintaining baseline synaptic activity as well as in the development of the *Drosophila* neuromuscular junction. In this chapter, I will first present my work in the context of the larger question of "How do we study complex genetic disorders?" I will then introduce the BLOC-1 complex, it's relevance to neurodevelopmental disorders, and it's role in basic cellular functions. I will focus on properties of synaptic vesicle organization, highlighting the known roles for BLOC-1 in the presynaptic compartment. I will briefly introduce the advantages of using *Drosophila melanogaster* as a model organism to address these questions. In doing so, I will answer the following central question at the core of my dissertation research:

> **How do genetic modifications affecting a defined protein interaction network, in this case defined by dysbindin-containing BLOC-1, regulate properties of synaptic vesicle release and plasticity which lead to complex behaviors?**

Section 1. Neurodevelopmental Disorders: Mechanisms and Boundary Definitions from Genomes, Interactomes, and Proteomes

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Neurodevelopmental disorders (NDD) are multifaceted conditions characterized by impairments in cognition, communication, behavior, and/or motor skills resulting from abnormal brain development. Intellectual disability, communication disorders, autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), motor disorders, and neuropsychiatric disorders such as schizophrenia fall under the umbrella of NDD (Reiss 2009, Rapoport, Giedd et al. 2012, American Psychiatric Association 2013). Currently, there are no biomarkers to diagnose NDD or to differentiate between them. Rather, these disorders are categorized into discrete disease entities based on clinical presentation (American Psychiatric Association 2013). This is problematic, as many symptoms are not unique to a single NDD, and several NDD have clusters of symptoms in common. For example, impaired social cognition is common to ASD and schizophrenia (King and Lord 2011, Korkmaz 2011, Sugranyes, Kyriakopoulos et al. 2011, Lugnegard, Unenge Hallerback et al. 2013) and psychosis is observed not only in schizophrenia but also in those with bipolar disorder or major depressive disorder (Domschke 2013, Owoeye, Kingston et al. 2013). Complicating the understanding of these disorders further is an overlapping set of genetic variations amongst NDDs, which prevents the use

of reliable genetic testing. Thus, the diffuse clinical boundaries and complex genetics of NDD present several challenges for examining the basic biological processes that these disorders are rooted in. Classical experimental assessments exploit the ease of studying monogenic disorders, where a single genetic variation is entirely responsible for the clinical presentation. However, in the case of NDDs, we have overlapping sets of both genetic variations, which frequently include numerous genes and gene products, and symptoms. How, then, do we go about studying these disorders in a directed fashion? Here, I will advocate for cultivating a more complete and inclusive understanding of the genetics and associated molecular pathways involved in these disorders, and present a case for using an experimentally curated interactome to guide our studies.

Neurodevelopmental Disorders: Boundary Definitions from Genomes

The hypothesis that NDD are distinct nosological entities predicts that genetic factors associated with risk for or causation of a given disorder should segregate with diagnostic categories; thus, in classical terms, there should be little or no overlap among the genetic factors implicated in each NDD. That is, the genes that operate in one disorder should not be involved in another. However, genetic epidemiology reveals substantive overlap between genes conferring risk for or causing NDDs.

Genetic defects associated with risk or causation of NDDs range from large chromosomal deletions to single nucleotide polymorphisms (SNPs). Notably, among major genomic defects, a number of chromosomal deletions are associated with intellectual disability, ASD and schizophrenia (Bassett, Scherer et

al. 2010, Doherty, O'Donovan et al. 2012, Malhotra and Sebat 2012, Moreno-De-Luca, Myers et al. 2013). Among the most frequent are 1q21.1, 16p11.2 and 22q11.2 (Karayiorgou, Simon et al. 2010, Moreno-De-Luca, Myers et al. 2013). The large number of genes affected by these deletions should cause little surprise that they give rise to disorders with overlapping phenotypes. However, smaller genetic modifications, specifically SNPs in non-coding regions, are shared among diverse NDDs (Smoller, Craddock et al. 2013). Genetic overlap among NDDs extends to monogenic defects that affect the coding sequence and expression of a single polypeptide encoded by the gene (e.g. *SHANK3, NRXN1, DISC1, FMR1, MECP2, GPHN)*. Patients carrying these mutations are diagnosed either with intellectual disability, ASD, schizophrenia, or combinations of these disorders (St Clair, Blackwood et al. 1990, Amir, Van den Veyver et al. 1999, Lam, Yeung et al. 2000, Cohen, Lazar et al. 2002, Chahrour and Zoghbi 2007, Kilpinen, Ylisaukko-Oja et al. 2008, Kirov, Gumus et al. 2008, Kirov, Rujescu et al. 2009, Ching, Shen et al. 2010, Calfa, Percy et al. 2011, Gauthier, Siddiqui et al. 2011, Porteous, Millar et al. 2011, Schaaf, Boone et al. 2012, Iqbal, Vandeweyer et al. 2013, Lionel, Vaags et al. 2013). Monogenic genetic defects affect subunits of obligate and stable protein complexes. For example, the adaptor complex AP-3 is an obligate heterotetramer that generates vesicles from early endosomes localized to lysosomes/synapses (Newell-Litwa, Seong et al. 2007, Larimore, Tornieri et al. 2011). Human mutations in a neuronal-specific AP-3 subunit (*AP3B2*) associate with ASD (Gokhale, Larimore et al. 2012, O'Roak, Vives et al. 2012). Thus, irrespective of the size of a genetic defect, there is a continuously expanding list

of affected genes that do not respect categorical diagnostic boundaries among NDDs.

Neurodevelopmental Disorders: Boundary Definitions from Interactomes

Protein interaction networks (interactomes) to which NDD genes belong also overlap. Interactomes built from genes associated with intellectual disability, ASD, ADHD, and schizophrenia converge on common molecular pathways (Cristino, Williams et al. 2013). Genes associated with these NDD intersect on one out of 700 genes catalogued as risk factors. However, the list of common proteins shared by these NDD increases to 147 out of the 700 genes simply by expanding the gene catalog to include predicted first-degree interacting neighbors obtained from protein-protein interaction databases (Cristino, Williams et al. 2013). These computational studies support the concept that the interactomes associated with NDD overlap. However, the power of these types of studies is limited by the present quality of protein interaction databases that are incomplete, are only moderately curated to accommodate new published findings, and are often populated by results not confirmed by alternative biochemical, genetic, and/or functional approaches (Guimera and Sales-Pardo 2009, Gokhale, Perez-Cornejo et al. 2012, Yu, Wallqvist et al. 2012). Furthermore, protein interaction databases are biased by the experimental approach used in their generation; for example, most protein interaction databases poorly represent membrane proteins that are not amenable to exploration by older techniques, such as traditional yeast two hybrid or pull-

downs with bacterial recombinant proteins, but we now have developed the tools to explore (Brito and Andrews 2011).

The interactome of the schizophrenia susceptibility gene *DTNBP1* well illustrates several of these problems (Figure 1). *DTNBP1* encodes dysbindin, a subunit of the BLOC-1 complex (Talbot, Eidem et al. 2004, Weickert, Rothmond et al. 2008, Ryder and Faundez 2009, Talbot, Ong et al. 2009, Tang, LeGros et al. 2009, Ghiani, Starcevic et al. 2010, Ghiani and Dell'Angelica 2011, Mullin, Gokhale et al. 2011, Talbot, Louneva et al. 2011). This complex participates in membrane protein trafficking between endosomes and lysosomes, and between endosomes located in neuronal cell bodies and the synapse (Ryder and Faundez 2009, Larimore, Tornieri et al. 2011, Mullin, Gokhale et al. 2011). Published in silico dysbindin interactomes (Li, Feng et al. 2007, Guo, Sun et al. 2009) differ from biochemically and genetically tested protein interaction networks (Gokhale, Larimore et al. 2012). However, discrepancies among interactomes expand beyond those published (Figures 1a and d). Three protein interaction databases report associations that differ from each other in interactor identities. Furthermore, feeding those associations into a rigorous algorithm for 'de novo' generation of interactomes reveals different network topologies (Figures 1a and d) (Rossin, Lage et al. 2011). Only one of these four dysbindin interactomes links dysbindin with the adaptor complex AP-3, despite multiple biochemical, cell biology, and genetic evidence that these complexes interact in vivo and in vitro (Figure 1a) (Di Pietro, Falcon-Perez et al. 2006, Salazar, Craige et al. 2006, Hashimoto, Ohi et al. 2009, Hikita, Taya et al. 2009, Newell-Litwa, Salazar et al.

2009, Oyama, Yamakawa et al. 2009, Taneichi-Kuroda, Taya et al. 2009, Newell-Litwa, Chintala et al. 2010). This deficiency in existing databases has immediate ramifications, as mutations in AP3B2 associated with ASD cannot be linked to schizophrenia through the BLOC-1 subunit dysbindin (Gokhale, Larimore et al. 2012, O'Roak, Vives et al. 2012). AP3B2 is not an isolated instance. Rather, only the experimentally defined dysbindin interactome identifies SNAP29 and CLTCL1 (Newell-Litwa, Salazar et. al, 2009). SNAP29 has been identified as a de novo risk factor for schizophrenia, while both SNAP29 and CLTCL1 map to the chromosome interval affected in velocardiofacial (chromosome 22q11.2 deletion) syndrome (Karayiorgou, Simon et al. 2010, Malhotra, McCarthy et al. 2011). This syndrome closely associates with schizophrenia, ASD, and intellectual disability (Karayiorgou, Simon et al. 2010). Gaps in content and quality in relation to protein interaction databases are important, as these repositories are the foundation for molecular connectivity between genetic defects associated with a given disorder. These deficiencies are missed opportunities for establishing molecular mechanisms of disease and finding mechanistic commonalities among NDDs. Thus, we argue in favor of generating interactomes confirmed by biochemical, genetic, and/or functional strategies. Epidemiological genomics offer the field a good selection of solid candidate genes with which to begin this quest.

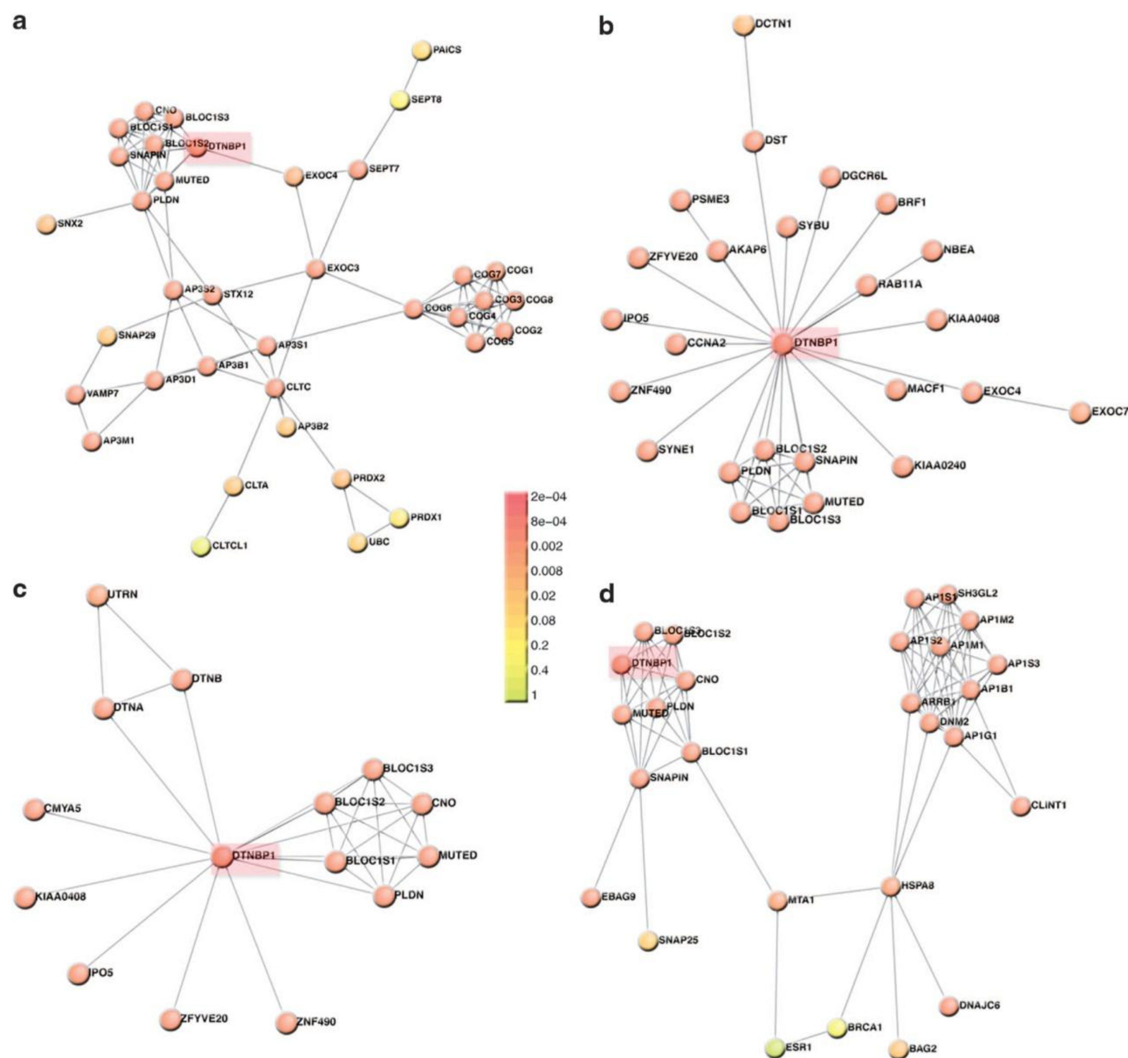

Figure 1. *DTNBP1*-dysbindin interactomes differ in their constituents and topology. Interactomes were assembled with the Dapple algorithm (http://www.broadinstitute.org/mpg/dapple/dapple.php) (Rossin, Lage et al. 2011) using as inputs the dysbindin associated proteins identified by affinity chromatography (A), and interactors reported in three protein-protein interaction databases: (B) Biogrid (http://thebiogrid.org/), (C) Genemania (http://www.genemania.org/) and (D) String 9.05 (http://string.embl.de/). Red boxes highlight *DTNBP1*. Note that the identity of interacting proteins differs among interactomes. Color code represents a Dapple estimated probability that a protein would be as connected to other proteins (directly or indirectly) by chance as is depicted. Only interactome A, presents a biochemically and genetically confirmed interaction between the adaptor complex AP-3 and the dysbindin-containing BLOC-1 complex

Neurodevelopmental Disorders "Guilty by Association" Mechanisms of Disease.

Loss of one protein function due to a genetic mutation can alter levels or activity of other proteins that interact either directly or indirectly with the mutant protein. Subunits of protein complexes are particularly susceptible to loss-of-function genetic modifications to interacting partners, making them "guilty by association" in the context of disease. Genetic defects, or even non-pathogenic allelic variation affecting a single subunit of a protein complex, frequently lead to down-regulation and/or covariation of other complex subunits (Kantheti, Qiao et al. 1998, Peden, Rudge et al. 2002, Li, Zhang et al. 2003, Starcevic and Dell'Angelica 2004, Jia, Gomez et al. 2010, Wu, Candille et al. 2013). *DTNBP1* null mutations preventing dysbindin expression down-regulate expression of most subunits of the BLOC-1 complex, despite the monogenic character of the mutation (Li, Zhang et al. 2003, Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011). Reciprocally, genetic defects on other BLOC-1 subunits decrease dysbindin cellular content (Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011). "Guilty by association" proteins in the dysbindin interactome extend beyond intrinsic components of the BLOC-1 complex. These proteins include membrane protein cargoes such as VAMP7 (*VAMP7*), a synaptic vesicle fusogenic membrane protein known as a v-SNARE (**v**esicular- **SNA**P **re**ceptor protein) implicated in spontaneous synaptic vesicle fusion, the Menkes disease copper transporter (*ATP7A*), the adaptor complex AP-3, RhoGEF1 (*ARHGEF1)*, and BDNF (*BDNF*), a neurotrophin with a long history of association with several NDD (Salazar, Craige et al. 2006, Newell-Litwa, Chintala et al. 2010, Hua, Leal-

Ortiz et al. 2011, Autry and Monteggia 2012, Ramirez and Kavalali 2012, Ryder, Vistein et al. 2013). None of these proteins whose levels are affected by mutations in *DTNBP1*, or other BLOC-1 complex subunits, can be identified in current protein interaction databases that focus on physical protein-protein interactions. This problem prevents their inclusion in any analysis seeking to connect genetic defects found in genome wide associations studies to relevant molecular pathology.

Creating Understanding from Genome Informed Proteomes-Interactomes.

Genome-wide association studies search clinically-defined patient populations for genetic markers that reach a threshold of statistical significance to associate with disease risk (Figure 2a). This approach encounters the problem that these disorders are polygenic and that categorical NDD definitions are not linked to biological markers or molecular phenotype (Gottesman and Shields 1967, Purcell, Wray et al. 2009). Thus, it is likely that genetically heterogeneous patient cohorts in these studies gather multiple molecular mechanisms of disease. However, these studies offer powerful insight when a particular genetic marker reaches statistical significance, despite the ‘noise’ introduced by the polygenic character of these disorders and the problems intrinsic to categorical NDD definitions. Genetic defects associated with one or multiple NDD should be seen as the tip of the iceberg to unravel biological mechanisms of disease. Interactomes of gene products consistently implicated in NDDs (‘tip of the iceberg genes’) are a fertile ground to search for disease mechanisms (Rossin, Lage et al. 2011, Luo, Huang et al. 2014). This prediction stems from the hypothesis that genomes of patients

affected by polygenic NDD should concentrate alleles that affect the expression or function of genes whose products belong to or modulate a relevant pathway (Sullivan 2012). We illustrate this concept in Figure 2b where gene-α has reached statistical significance in a population GWAS. The product encoded by gene-α is a bait to 'fish out' the red protein interaction network (Red interactome B1, Figure 2b). The biochemical definition of interactome 1 would occur irrespective of whether interactome 1 contains products encoded by genes carrying defects that do not cross a population statistical threshold (Figure 2b).

This genome to proteome 'reverse' approach is not foreign to current genomic studies, in which bioinformatics of protein–protein interaction databases are used to find connections between gene defects that associate with a disorder at a GWAS level (O'Roak, Vives et al. 2012, Luo, Huang et al. 2014) (Figure 2c). However, mapping GWAS results back to an interactome requires the availability of several network genes that cross a statistical threshold (Red interactome 1, Figure 2c) as well as pre-existing and reliable protein interaction databases. Genes below statistical threshold in the red network C1 would not contribute to the identification of the C1 interactome (Red interactome C1, Figure 2c). Moreover, current criteria to allocate GWAS results to an interactome would miss the yellow interactome C2 where genes encoding interactome products are all below statistical threshold (Figure 2c).

How can we obtain mechanistic insight from studying 'omes'? We propose two non-exclusive approaches to define the biology of NDDs using protein–protein interaction networks and genomics. The first approach is through the definition

of ‘tip of the iceberg gene’ protein networks, such as those depicted by the red interactomes in Figures 2b and c. Second, reliable protein interactomes can be used as a query matrix to explore patient’s genomes for genetic defects or variants targeting interactome encoding loci. Different patients may carry defects in one or more genes encoding products belonging to an interactome. Each gene defect does not reach statistical significance in a ‘gene-centric’ GWAS study (Subject 1–3, Figure 2d).

However, collective analysis of the genomes in a cohort of patients (Subject 1–3, Figure 2d) shows significant enrichment of genetic defects clustered on a common pathway (compare red and yellow interactomes, Figure 2e). The association of a biological mechanism, defined by an already known and reliable interactome, with the genome of affected individuals would occur, although each gene in isolation would have not risen above statistical threshold. In this case, statistical significance is assigned to a collection of genes defining an interaction network rather than a single gene (Figure 2e).

These solutions depend on reliable protein interactions networks. As mentioned above, the quality of protein–protein interaction databases commonly used is substandard. This is due to a lack of thorough biochemical, functional and/or genetic confirmation of interactions. We posit that it is possible to extract more information about disease mechanisms and disorder boundaries from current GWAS studies if reliable protein interaction maps were to exist. As these are either not available or they are in construction, we propose to focus efforts on

defining the interactomes of (a) NDDs 'tip of the iceberg genes' as well as (b) 'guilty by association' proteins detected in the proteomes of cells carrying genetic defects in 'tip of the iceberg genes'. These and other experimentally confirmed interactomes (yellow interactome 2 in Figure 2e) would allow us to extract novel genetic information from existing and future GWAS.

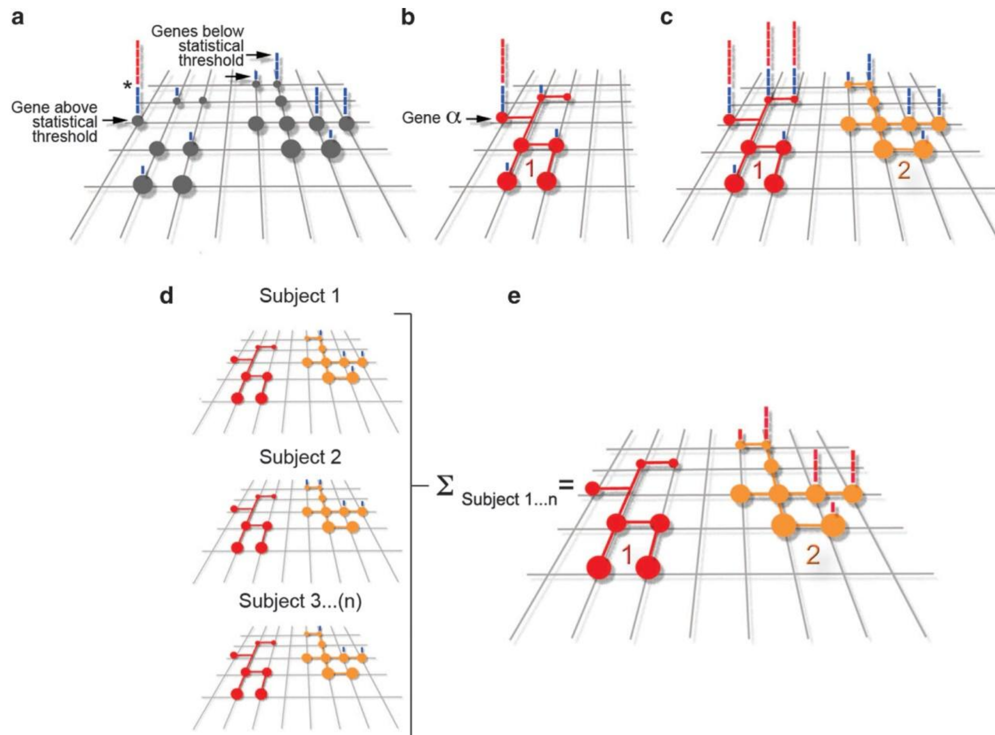

Figure 2. Models of cross-fertilization between genomes, proteomes, and interactomes. Grid in diagrams A to E depicts a polygenic genetic landscape associated to a NDD. Circles represent defined genes within the grid that when affected in different combinations trigger a NDD. Bars above each gene indicate a subject where a gene defect was found on a GWAS. Blue bars are those subjects that have a defect in a gene below statistical threshold, which is marked by the asterisk in A. Red bars above a gene represent subjects that have a defect in a gene above statistical threshold. B depicts a 'tip of the iceberg gene α' and the network to which it belongs represented by the connected red circles (interactome 1). C depicts three 'tip of the iceberg gene' and the network to which they belong (interactome 1). The yellow interactome 2 is constituted by genes below statistical threshold as defined by gene-centric GWAS statistical analysis. D represents genetic defects (blue bars) in two interactomes per patient (subjects 1-3). Note that in all patients there is no gene defects in the red interactome. E depicts hypothetical results of an interactome-centric GWAS that includes subjects 1-3 in D. The yellow interactome 2 is now above statistical threshold as defined by an interactome-centric GWAS statistical analysis. See text for details.

Creating a genome-independent nosology from proteomes-interactomes

Human proteomes are hereditable molecular phenotypes(Wu, Candille et al. 2013) and as such constitute valuable, yet untapped, resources to create disorder classifications rooted in molecules and their pathways. The study of proteomes shares with the analysis of genomes its quantitative and unbiased character. However, proteomes and interactomes offer the distinctive advantage of being executors of phenotypic programs in cells and tissues. Therefore, proteomes and interactomes are causally closer to the identity of disease mechanisms than genomes. Proteomes are already beginning to shed light on complex neurological disorders such as schizophrenia (English, Pennington et al. 2011, Martins-de-Souza 2012). However, we should not limit ourselves to just exploring postmortem brains of subjects grouped solely by their clinical features. Instead, we advocate for the study of proteomes from cells isolated from individuals that are genetically related. Cell proteomes from affected probands compared with their unaffected first-degree relatives offer a great prospect for the identification of hereditable or de novo abnormalities in molecular phenotypes. Evidently, in the context of NDDs, human inducible pluripotent stem cells are a great resource, as they can be differentiated into neurons (Goldstein 2012). However, it is likely that the molecular mechanisms affected in NDDs are common to many, if not all cells. For example, Fragile X syndrome or velocardiofacial syndrome, where multiple tissues are affected (Moreno-De-Luca, Myers et al. 2013). Thus, fibroblasts or lymphoblasts from human pedigrees are likely to offer valuable insights into neuronal disorders. We predict that proteomes built from

genetically related subjects' cells will bridge two camps. On one hand, proteomes will help us to interpret results from genome-wide analyses. On the other hand, they will guide us to define NDD mechanisms at levels of complexity higher than the traditional single genes or proteins. These would include, for instance, subcellular compartments, such as synapses or mitochondria, and deficits in tissue organization, such as those in neural circuits. Genomes, proteomes and interactomes give us vantage points, the inevitable next step is to dive deep into the biology emerging from and converging to them.

My dissertation research is founded on a biochemically curated protein interaction network defined by the BLOC-1 subunit dysbindin. This network identified the seven remaining BLOC-1 subunits as being down-regulated after genetic perturbation to dysbindin. Additionally, several factors outside of BLOC-1 were identified in this interactome. In this work, I will examine the complexities of the interactions within the complex, largely using the *Drosophila* neuromuscular junction to measure synaptic read-outs, as well as to use this curated network to test the hypothesis that proteomes and interactomes can guide us in defining mechanisms of NDDs. While these interactions do arise from this dysbindin-derived interactome, the focus of the work is on the function of the entire BLOC-1 complex, and how BLOC-1 complex network stability is essential for normal synaptic functions. In the following chapter, I provide a comprehensive description of the BLOC-1 complex biochemistry and cellular functions, as well as the role of the BLOC-1 complex in synaptic biology and association with schizophrenia, a neurodevelopmental disorder.

Section 2. Cell Biology of the BLOC-1 Complex Subunit Dysbindin, a Schizophrenia Susceptibility Gene.

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Association of dysbindin and schizophrenia: Genetic evidence

A central feature of schizophrenia is the strong genetic component associated with disease development as concluded from monozygotic twin studies, which indicate a heritability of ~80% (Cannon, Kaprio et al. 1998, Cardno, Marshall et al. 1999, Sullivan, Kendler et al. 2003). Rare highly penetrant genetic deficiencies have lead to possible disease mechanisms. These include copy number variations such as chromosome microdelections or microduplications (Consortium 2008, Stefansson, Rujescu et al. 2008, McCarthy, Makarov et al. 2009, Ingason, Rujescu et al. 2010, Karayiorgou, Simon et al. 2010, Moreno-De-Luca, Mulle et al. 2010, Mulle, Dodd et al. 2010); chromosomal translocations comprising the *DISC1* locus (Brandon, Millar et al. 2009); and mutations affecting the post synaptic scaffolding protein Shank3 (Gauthier, Champagne et al.) or a kinesin motor isoform (Tarabeux, Champagne et al. 2010). In contrast to these genetic variants, the vast majority of schizophrenia cases fit into a polygenic model where principal contributions to disease are believed to result from convergence of multiple genes of small to moderate effect size (Gottesman and Shields 1967, Risch 1990, Purcell, Wray et al. 2009). These genetic features of schizophrenia have hampered the progress in the understanding of disease pathogenesis.

Genome-wide analyses of schizophrenia-affected individuals have uncovered multiple haplotypes that strongly associate with disease. In these studies *DTNBP1* ranks 20th in a group of 45 genes selected as strongly associated with disease risk out of a total pool 1008 genes studied thus far (Allen, Bagade et al. 2008, Schizophrenia_Research_Forum 2010). For example, a defined allele in the *DTNBP1* gene (rs1011313) was associated with susceptibility to schizophrenia in a meta-analysis comparing 2,696 Caucasian patients with schizophrenia with 2,849 controls (Allen, Bagade et al. 2008, Schizophrenia_Research_Forum 2010). Based on the Ioannidis guidelines for the analysis of cumulative evidence in genetic association studies, the *DTNBP1* association with disease is considered with a strong degree of epidemiologic reliability (Ioannidis, Boffetta et al. 2008). However, attempts to identify mutations in the exome of *DTNBP1* in schizophrenia patients have been negative so far (Dwyer, Carroll et al.). Moreover, the only human case reported carrying a loss-of-function allele in *DTNBP1* lacked psychiatric manifestations. This patient carried a homozygote 307C-T transition in the *DTNBP1* gene resulting in the substitution of glutamine 103 to a stop codon (Q103X). The patient, the daughter of consanguineous parents, was affected by Hermansky-Pudlak syndrome type 7 (HPS7; OMIM 203300) exhibiting oculocutaneous albinism, ease of bruising, and a bleeding tendency, yet there were “no apparent behavioral abnormalities in the [affected] individual” nor a report of disease in her consanguineous relatives (Li, Zhang et al. 2003). The absence of evidence of psychiatric illness in this patient, her family, and an individual affected by Hermansky-Pudlak type 8 (HSP8; OMIM 203300; see below), raise doubts about the involvement of *DTNBP1* in disease.

This uncertainty about *DTNBP1* in disease susceptibility is further enhanced by the inconsistent association of defined *DTNBP1* polymorphisms with schizophrenia (Straub, Jiang et al. 2002, Schwab, Knapp et al. 2003, Williams, Preece et al. 2004) and the inconsistent association of the *DTNBP1* locus with disease across multiple patient cohorts of diverse ethnicities (Morris, McGhee et al. 2003, Mutsuddi, Morris et al. 2006, Turunen, Peltonen et al. 2007, Peters, Wiltshire et al. 2008, Strohmaier, Frank et al. 2010). However, it should be kept in perspective that even when the genome between individuals is identical, as is the case with monozygotic twins, not all individuals develop schizophrenia arguing for other factors necessary to trigger disease. The polygenic character of schizophrenia raises the issue that individuals possessing susceptibility alleles in genes, such as *DTNBP1*, may express psychiatric phenotypes only when these genetic variants occur in a propitious genome and when environmental factors come to play (Abazyan, Nomura et al. 2010). A propitious genome for disease development would contain additional susceptibility alleles in other loci. Each allele in isolation would not trigger disease. However, only a combination of susceptibility alleles in different genetic loci would trigger disease.

Biochemical, anatomical, and functional consequences of carrying *DTNBP1* polymorphisms associated to disease.

The genetic evidence for the involvement of *DTNBP1* in schizophrenia may be considered ambiguous. However, a stronger case about *DTNBP1* involvement on disease emerges when considering molecular, anatomical, and systems/behavioral phenotypes associated with *DTNBP1* polymorphisms in humans.

DTNBP1 polymorphisms associated with disease are found in non-coding regions of the *DTNBP1* locus (Guo, Sun et al. 2009). However, the reduced levels of expression of *DTNBP1* mRNA and protein in post-mortem schizophrenia brains suggest that these non-coding polymorphisms could affect transcript or protein levels. Pioneer quantitative immunocytochemistry and immunoblot studies by Talbot indicate that 73 to 93% of schizophrenia cases displayed dysbindin reductions in the hippocampal region. Reductions averaged 18 to 42% in two clinical case cohorts totaling 32 patients (Talbot, Eidem et al. 2004). These findings contrast with the normal levels of the synaptic vesicle protein synaptophysin in the same cases arguing against a loss of synapses. These findings have been replicated using biochemical analysis of dysbindin levels in homogenates of the dorsolateral prefrontal cortex of schizophrenic patients in two independent studies (Talbot, Eidem et al. 2004, Tang, LeGros et al. 2009, Talbot, Louneva et al. 2011). Reduction in hippocampal protein levels is mirrored by reduced levels of dysbindin mRNA in the hippocampal formation of patients with schizophrenia (Weickert, Straub et al. 2004, Bray, Preece et al. 2005, Weickert, Rothmond et al. 2008). Similarly, patients with schizophrenia had decreased dysbindin mRNA levels in multiple layers of the dorsolateral prefrontal cortex, whereas synaptophysin mRNA levels seem unaffected (Weickert, Straub et al. 2004). Importantly, *DTNBP1* polymorphisms associated with increased disease risk may influence the content of *DTNBP1* messenger RNA (Bray, Preece et al. 2005). *DTNBP1* polymorphisms associated to increased schizophrenia risk correlate with a reduction in *DTNBP1* mRNA expression in human cerebral cortex whereas putative ‘protective’ polymorphisms associate with high *DTNBP1*

expression (Bray, Preece et al. 2005). Although these observations need replication in other patient cohorts, they provide good circumstantial evidence supporting the hypothesis that non-coding polymorphisms in *DTNBP1* regulate its transcript expression and thus dysbindin content and function in specific areas of the brain.

DTNBP1 risk polymorphisms associated with reduced levels of mRNA in patients also correlates with reduced gray matter volume in the dorsolateral prefrontal and occipital cortex (Donohoe, Frodl et al. 2010). These observations well fit well to the behavioral findings of impaired spatial working memory (Donohoe, Morris et al. 2007) and electrophysiological data consistent with impaired visual processing also observed in carriers of this same *DTNBP1* risk polymorphisms (Donohoe, Morris et al. 2008). Reduced volume of grey matter has been recapitulated in other patient cohorts with a different *DTNBP1* risk polymorphism (Narr, Szeszko et al. 2009) further supporting the model that *DTNBP1* susceptibility variants may affect specific cortical regions in schizophrenia. Furthermore, evidence is mounting supporting the idea that *DTNBP1* risk polymorphisms associate with differences in brain function encompassing changes in prefrontal brain function (Fallgatter, Herrmann et al. 2006), cortical activation during verbal tasks (Markov, Krug et al. 2009), visual processing (Donohoe, Morris et al. 2008, Mechelli, Viding et al. 2010), and emotional working memory (Wolf, Jackson et al. 2009). Collectively, correlative biochemical, genetic, anatomical and functional evidence argues that carriers of *DTNBP1* alleles associated with disease display phenotypes of progressive

complexity that may contribute to schizophrenia susceptibility. Unraveling the contributions of the *DTNBP1* locus to schizophrenia requires a comprehensive understanding of phylogenetically conserved dysbindin molecular interactions, the fundamental cellular pathways modulated by dysbindin and its associated proteins, and cell-cell interactions influenced by dysbindin and its interactors. These fundamental questions are the central focus of this manuscript.

The Eight Musketeers: "all for one, one for all", that is BLOC-1's motto.

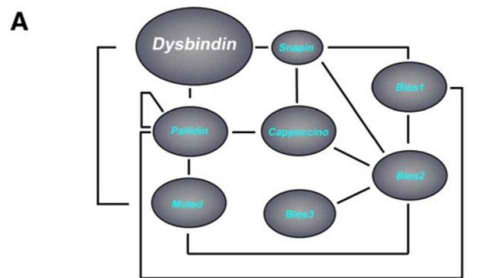
A common occurrence in the psychiatric literature related to dysbindin is to consider this protein in isolation. Instead genetics and comparative phylogeny define a conserved core of dysbindin molecular interactions. These dysbindin interactions define a stable protein complex known as the BLOC-1 complex (Biogenesis of Lysosome-related Organelles Complex-1). Most brain dysbindin, if not all, exists as part of BLOC-1 a large molecular weight complex with a Stokes' radius of ~95 Å and a native molecular size of 200 ± 30 kDa. BLOC-1's molecular weight far exceeds dysbindin's predicted molecular weight of ~39.5 kDa (Starcevic and Dell'Angelica 2004, Ghiani, Starcevic et al. 2010). The BLOC-1 complex is a heterooctamer comprising of dysbindin, pallidin, muted, cappuccino, BLOS1, BLOS2, BLOS3/reduced pigmentation and snapin polypeptides (Di Pietro and Dell'Angelica 2005) (Figure 3A. Figure 3B shows three BLOC-1 subunits coprecipitating with recombinant dysbindin). The molecular architecture of binary interactions between BLOC-1 complex subunits is conserved from *Drosophila* to mammals as determined by two-hybrid analysis (Starcevic and Dell'Angelica 2004, Di Pietro and Dell'Angelica 2005, Nazarian,

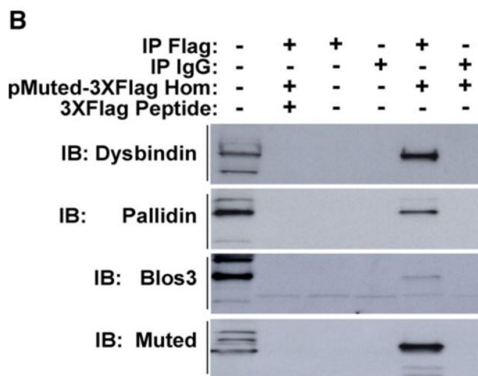
Starcevic et al. 2006, Cheli, Daniels et al. 2010) (Figure 3A depicts interactions among BLOC-1 subunits in mammals). This illustrates that the BLOC-1 organization was selected early on in evolution and its conservation from fly to human argues for the functional significance of this architecture. The phenotype of pallidin, muted, BLOS3/reduced pigmentation, cappuccino, and dysbindin deficient mice further supports dysbindin incorporation into this complex. First, loss of one BLOC-1 subunit triggers down-regulation of other complex subunits. For example, dysbindin-null mice *sandy* ($DTNBP1^{sdy/sdy}$) express reduced levels of muted, pallidin, and snapin polypeptides. For example, Figure 4 illustrates the absence of pallidin in $Pldn^{pa/pa}$ mouse hippocampus, a phenotype similarly observed in $DTNBP1^{sdy/dy}$ hippocampus. Concurrently, dysbindin is reduced in null mouse models of Blos3/reduced pigmentation ($BLOC1S3^{rp/rp}$), muted ($Muted^{mu/mu}$) and pallid ($Pldn^{pa/pa}$) (Li, Zhang et al. 2003, Starcevic and Dell'Angelica 2004, Feng, Zhou et al. 2008).

These molecular/genetic associations and biochemical phenotypes of loss-of-function alleles converge again in the systemic phenotypes of pallidin, muted, BLOS3/reduced pigmentation, cappuccino and dysbindin deficient mice. They all share phenotypes including oculocutaneous pigment dilution, bleeding diathesis, and pulmonary fibrosis that are hallmarks of a human autosomal recessive disorder –the Hermansky-Pudlak syndrome (Li, Rusiniak et al. 2004, Di Pietro and Dell'Angelica 2005, Wei 2006). A phenotypic trait –pigment dilution- is also observed in flies carrying null mutations in Blos1 (CG30077)(Cheli, Daniels et al. 2010). Similarly, patients with mutations in dysbindin (Hermansky-Pudlak

syndrome type 7, HPS7) and Blos3/reduced pigmentation (Hermansky-Pudlak syndrome type 8, HPS8) share all or part of the phenotypic triad found in BLOC-1-deficient mice (Li, Zhang et al. 2003, Morgan, Pasha et al. 2006). Together, these phenotypes from model genetic organisms and humans, strongly support a role for dysbindin in functions integral to the BLOC-1 complex.

Interestingly, and somewhat surprisingly, there are no reports of neuropsychiatric phenotypes in the two patients bearing mutations in BLOC-1 subunits: dysbindin (HPS7) and Blos3/reduced pigmentation (HPS8)(Li, Zhang et al. 2003, Morgan, Pasha et al. 2006). In contrast, mice and flies lacking





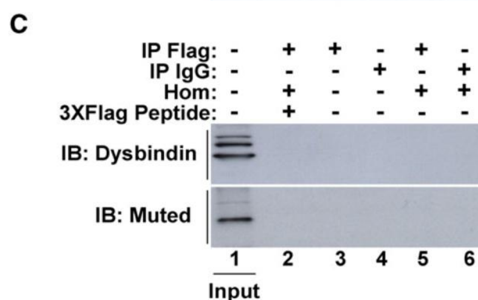


Figure 3. Molecular Architecture of the BLOC-1 complex. A) Diagram represents the molecular associations between the eight subunits of the BLOC-1 complex as determined from yeast two hybrid analyses and information in curated databases (http://thebiogrid.org/) (Starcevic and Dell'Angelica 2004, Di Pietro and Dell'Angelica 2005, Cheli, Daniels et al. 2010) B) Dysbindin Coimmunoprecipitates with BLOC-1 subunits. Cell extracts from HEK293 cells expressing muted tagged with a triple Flag tag (B) or untransfected (C) were immunoprecipitated (IP) with beads coated with Flag antibodies (lanes 1, 2, and 5) or control mouse IgG (lane 4 and 6). As controls, immunoprecipitations were outcompeted with triple Flag peptide to prevent binding of muted Flag and associated proteins to beads (lane 2) or homogenates (Hom) were excluded from reactions carrying Flag antibodies (Lane 3). The presence of the BLOC-1 subunits dysbindin, pallidin, and Blos3 was determined by immunoblot of SDS-PAGE

BLOC-1 subunits possess well-defined neurological phenotypes. There is a growing literature documenting neuro-behavioral phenotypes in *sandy* mice (*DTNBP1*$^{sdy/dy}$)(Talbot 2009) despite the fact that the initial description of the *sandy* mutation did not detect neurobehavioral abnormalities (Li, Zhang et al. 2003). Deficiencies in the dysbindin and Blos1 *Drosophila* orthologues trigger synaptic electrophysiological phenotypes and neuro-behavioral abnormalities (Dickman and Davis 2009, Cheli, Daniels et al. 2010). Similarly, dysbindin and snapin null mice share common synaptic electrophysiological phenotypes (see below). Biochemically defined synaptic vesicle and hippocampal phenotypes are also shared in three BLOC-1 null mutations namely *muted*, *pallid* and *sandy* (Larimore unpublished observations and (Newell-Litwa, Salazar et al. 2009, Newell-Litwa, Chintala et al. 2010)). This phenotypic convergence is extended to behavioral phenotypes shared in *muted*, *pallid*, *reduced pigmentation*, and *cappuccino* deficient mice (Newell-Litwa, Chintala et al. 2010). Collectively, these results from *Drosophila* and mice argue for a phylogenetically conserved role of BLOC-1 in synaptic function. Significantly, studies by Talbot, in brain tissue samples from schizophrenia patients, point to an association of dysbindin reduction and synaptic vesicle composition as exemplified by the increased level of the vesicular glutamate transporter 1, (Vglut1) in presynaptic terminals of schizophrenia brains (Talbot, Eidem et al. 2004). Importantly, Vglut1 forms a complex with BLOC-1 in brain synaptic fractions and neuronal cell lines (Newell-Litwa, Chintala et al. 2010).

It is reasonable to hypothesize that a role for dysbindin in the risk of schizophrenia pathogenesis is as an integral subunit of the BLOC-1 complex. This hypothesis makes two predictions. First, alleles in BLOC-1 subunit genes should influence disease risk. In fact, significant association between *BLOC1S3* alleles and schizophrenia and epistatic genetic interactions between *DTNBP1* and *MUTED* contributing to schizophrenia support the first prediction (Morris, Murphy et al. 2008). However, a genetic association between *DTNBP1* and *MUTED* in schizophrenia patients is controversial (Gerrish, Williams et al. 2009). Secondly, reduction of dysbindin protein in schizophrenia should be associated with reduced levels of other BLOC-1 proteins. Among those proteins, Talbot has found reductions not only in dysbindin, but also in pallidin, snapin, and especially BLOS3 in the inner molecular layer of the dentate gyrus of schizophrenia cases (Talbot, personal communication). Similarly, we have observed reduced levels of pallidin in the hippocampal formation of sandy mice (Fig. 4). These are suggestive leads that need further development to properly implicate the BLOC-1 complex in schizophrenia and other psychiatric disorders.

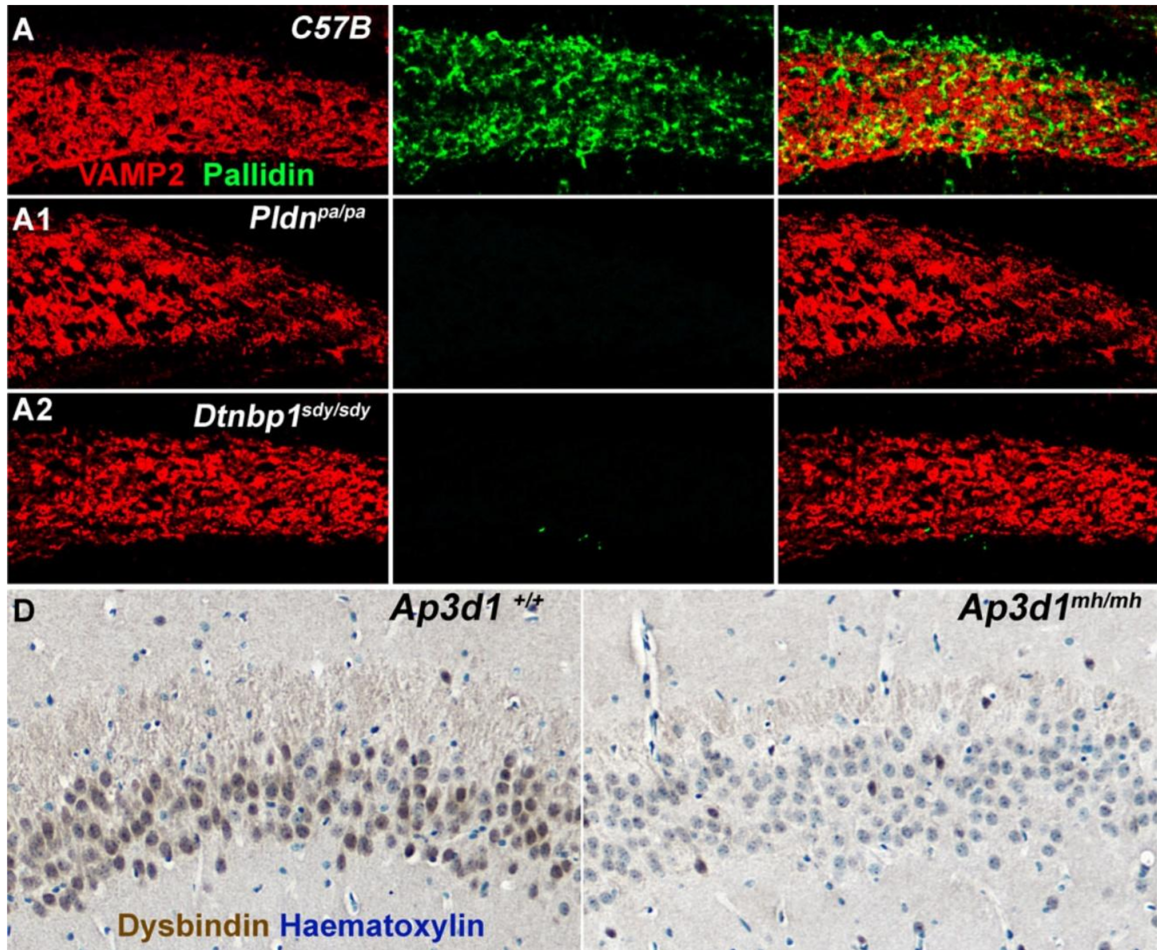


Figure 4. Levels of the BLOC-1 subunits pallidin and dysbindin in brains of wild type and BLOC-1 or AP-3 mutant mice. (A) Depicts double immunofluorecent confocal microscopy images of the dentate gyrus from wild type (C57B) and BLOC-1 null *sandy* (*DTNBP1*$^{sdy/sdy}$) and *pallid* (*Pldn*$^{pa/pa}$) mice stained with antibodies against the synaptic vesicle SNARE VAMP2 (red channel) and the BLOC-1 subunit pallidin (green channel). Note the absence of the BLOC-1 subunit pallidin both in *pallid* mouse and the dysbindin null *sandy* mouse. (B) Images of the CA1 region of the hippocampus from wild type (*Ap3d1*$^{+/+}$) and mocha (*Ap3d1*$^{mh/mh}$) mice stained with antibodies against dysbindin. Antibody-antigen complexes were revealed with an immunoperoxidase reaction. Note the reduction in the levels of dysbindin in AP-3 null *mocha* hippocampal tissue.

What fundamental cellular processes are affected in dysbindin/ BLOC-1 loss-of-function?

There is an expansion in the reported interactions and putative functions of dysbindin and its complex, BLOC-1 (Li, Feng et al. 2007, Guo, Sun et al. 2009, Rodriguez-Fernandez and Dell'Angelica 2009, Mead, Kuzyk et al. 2010). However, we are lacking quantitative and unbiased identification of the molecular interactors of dysbindin/BLOC-1 such as those offered by genetic screens and quantitative proteomics. Definition of stoichiometric dysdindin/BLOC-1 interactors is needed to prioritize them and assess their potential impact on the dysbindin/BLOC-1 loss-of function phenotypes. We discuss these interactions beginning this section with promising emerging data hinting to roles of dysbindin-BLOC-1 on transcriptional and cytoskeletal regulation. We finish discussing BLOC-1/dysbindin interactions relevant to membrane protein sorting and membrane fusion/secretion, which are the best-documented as defined either by data replication or by the convergence of multiple biochemical and genetic approaches.

Transcriptional and Cystoskeletal Regulation

The roles of BLOC-1 subunits in transcriptional and cytoskeletal regulation are not yet thoroughly defined. Transcriptional control by dysbindin is suggested by reports of a nuclear localization of this protein (Oyama, Yamakawa et al. 2009, Fei, Ma et al. 2010, Okuda, Kuwahara et al.). Dysbindin binds the nuclear transcription factor Y beta (NF-YB) and modulates transcription via this protein interaction. Similarly, the entry of dysbindin into the nucleus has been proposed to increase transcription of synapsin I gene and synapsin I protein content

(Numakawa, Yagasaki et al. 2004, Fei, Ma et al. 2010). Synapsin I in turn reversibly tethers synaptic vesicles to the actin cytoskeleton (Cesca, Baldelli et al. 2010). Synapsin I transcript and protein are decreased in the cortex and hippocampal formation of dysbindin-null mice (Fei, Ma et al. 2010). Whether these transcriptional effects of dysbindin occur in the context of BLOC-1 complexes or reflect the isolated activity of dysbindin remains unknown. Since deficiencies in other BLOC-1 subunits, Blos3/reduced pigmentation (*BLOC1S3*$^{rp/rp}$), muted (*Muted*$^{mu/mu}$) and pallid (*Pldn*$^{pa/pa}$), reduce the cellular levels of dysbindin (Li, Zhang et al. 2003, Starcevic and Dell'Angelica 2004, Feng, Zhou et al. 2008), it is plausible that the levels of synapsin I are also down-regulated in other BLOC-1 deficiencies. These changes in the content of transcripts likely reflect the tip of the iceberg and unbiased analyses of the transcriptome of BLOC-1 deficient brains are needed to fully uncover the potential of dysbindin and BLOC-1 in regulating transcription.

It is interesting that dysbindin controls the levels of synapsin I, a protein with capacity to bind and bundle actin filaments (Cesca, Baldelli et al. 2010). The activity of dysbindin controlling actin dynamics may extend beyond transcriptional control of an actin-binding protein such as synapsin I. Dysbindin interacts with actin regulatory proteins WAVE2 and Abi1 (Ito, Morishita et al.) and pallidin co-sediments with polymerized actin (Falcon-Perez, Starcevic et al. 2002). Down-regulation or the absence of dysbindin alters the architecture of actin cytoskeleton in neuroblastoma cells and in growth cones of dysbindin-null cultured hippocampal neurons (Kubota, Kumamoto et al. 2009). Moreover,

dysbindin siRNA alter the morphology of dendritic spines, a phenotype postulated to involve local alterations of the actin cytoskeleton (Ito, Morishita et al.). To what extent these growth cone and dendritic spine phenotypes are due to contribution of other cellular mechanisms, such as transcription, is presently unknown. Whether these effects of dysbindin on the actin cytoskeleton are either functional properties of the BLOC-1 complexes or the isolated activity of dysbindin has not been resolved.

Membrane Protein Sorting

The first suggestion that BLOC-1 may play a role in membrane protein sorting came from the phenotypic similarities between BLOC-1 deficiencies and loss-of-function alleles affecting subunits of the clathrin-adaptor complex 3 (AP-3). AP-3 is a heterotetramer constituted by δ, β3A or β3B, μ3A or μ3B, and σ3A or σ3b subunits (Newell-Litwa, Seong et al. 2007, Dell'angelica 2009). AP-3 recognizes sorting signals in the cytosolic domain of selected membrane proteins destined from endosomes to lysosomes, lysosome-related organelles -such as melanosomes and platelet dense granules- and synaptic vesicles (Bonifacino and Traub 2003, Robinson 2004, Newell-Litwa, Seong et al. 2007). Like other clathrin adaptors, AP-3 orchestrates the concentration of membranes proteins into nascent vesicles destined to these locations as well as deformation of the lipid bilayer to generate a vesicle (Bonifacino and Traub 2003, Robinson 2004). The precise role that BLOC-1 may play in membrane protein sorting and vesiculation is not clear, yet substantial evidence support a shared role of BLOC-1 and AP-3 in sorting and vesiculation processes.

AP-3 and BLOC-1 deficiencies possess common phenotypes in mouse and humans triggering the diagnostic triad that characterizes Hermansky-Pudlak syndrome. Deficiencies in the human *Ap3b1* locus, encoding the β3A polypeptide, lead to Hermansky-Pudlak syndrome type 2 (HPS2). Natural and engineered mouse mutants affecting the AP-3 subunits δ or β3A recapitulate main features of human HPS2 (Di Pietro and Dell'Angelica 2005, Newell-Litwa, Seong et al. 2007). The close association between BLOC-1 and AP-3 is further highlighted by the reduced immunoreactivity of AP-3 in the dentate gyrus of BLOC-1 deficient mice muted ($Muted^{mu/mu}$) and pallid ($Pldn^{pa/pa}$) (Newell-Litwa, Chintala et al. 2010) and the decreased dysbindin immunostaining in the dentate gyrus of AP-3 null mocha mice ($Ap3d1^{mh/mh}$, see Figure 2) (Salazar, Craige et al. 2006). Predictably, AP-3 or BLOC-1-deficiency phenotypic similarities are shared at a cellular level where a hallmark phenotype is increased cell surface levels of membrane proteins known to bind to AP-3 (Le Borgne, Alconada et al. 1998, Janvier and Bonifacino 2005, Di Pietro, Falcon-Perez et al. 2006, Salazar, Craige et al. 2006, Setty, Tenza et al. 2007, Baust, Anitei et al. 2008). Interestingly, this phenotype has been reported for dopamine D2 receptors (DRD2) and NMDA receptors in neuronal cells lacking or down-regulated for pallidin, muted or dysbindin BLOC-1 subunits (Iizuka, Sei et al. 2007, Ji, Yang et al. 2009, Tang, Yang et al. 2009, Marley and von Zastrow 2010). The phenotypic similarities extend to regulated secretory organelles where the absence of the BLOC-1 subunits dysbindin or snapin leads to enlarged chromaffin granules and synaptic vesicles, phenotypes in part recapitulated in AP-3 null mice ($Ap3d1^{mh/mh}$) (Grabner, Price et al. 2006, Asensio, Sirkis et al. 2010, Newell-Litwa, Chintala et

al. 2010). The commonality of systemic and cellular phenotypes between AP-3 and BLOC-1 loss-of-function mutants is explained in part by the coexistence of BLOC-1 and AP-3 on the same vesicles (Salazar, Craige et al. 2006, Newell-Litwa, Salazar et al. 2009), a reflection of a biochemical interaction between BLOC-1 an AP-3 (Di Pietro, Falcon-Perez et al. 2006, Salazar, Zlatic et al. 2009, Newell-Litwa, Chintala et al. 2010). In fact, the interaction between dysbindin and/or BLOC-1 with the adaptor complex AP-3 has been documented independently by ten publications (Di Pietro, Falcon-Perez et al. 2006, Salazar, Craige et al. 2006, Hashimoto, Ohi et al. 2009, Hikita, Taya et al. 2009, Newell-Litwa, Salazar et al. 2009, Oyama, Yamakawa et al. 2009, Salazar, Zlatic et al. 2009, Taneichi-Kuroda, Taya et al. 2009, Mead, Kuzyk et al. 2010, Newell-Litwa, Chintala et al. 2010).

What role does BLOC-1 play once on membranes? A plausible hypothesis is that BLOC-1 could act as adaptor/accessory adaptor recognizing membrane protein cargoes. Adaptors are recruited to membranes by the action of small GTP binding proteins of the ras superfamily and phosphoinositol phospholipids (Bonifacino and Glick 2004). Arf GTPases recruit clathrin adaptors. Such is the case of AP-3, which is recruited to endosome membranes by Arf1 in its GTP conformation (Faundez, Horng et al. 1998, Ooi, Dell'Angelica et al. 1998). However, whether BLOC-1 is recruited to membranes by Arf-dependent mechanisms is not yet defined. Suggestive data indicate that the association of AP-3 with the BLOC-1 complex in membranes is enhanced by the addition of non-hydrolysable GTP analogues (Di Pietro, Falcon-Perez et al. 2006). Whether this represents BLOC-1

independently being recruited by Arf GTPases to endosomal membranes or BLOC-1 'piggybacking' on the Arf-dependency of an AP-3 membrane recruitment mechanism remains to be resolved. Once recruited to membranes by Arf family members, adaptors, and accessory adaptors recognize sorting signals in the cytosolic domain of membrane proteins (cargoes) and concentrate them into nascent vesicles (Bonifacino and Traub 2003, Bonifacino and Glick 2004, Robinson 2004). Vesicles bud off and shed their attached coat (Di Pietro, Falcon-Perez et al. 2006).

A predictable consequence of an adaptor/accessory adaptor deficiency is the absence of cargoes in target organelles. This could result from to either a vesicle carrier not being made or a family of membrane proteins not being included in a vesicle. BLOC-1 can be found in vesicles coated with AP-3 or clathrin-coated vesicles both in neuronal and non-neuronal cells lines, suggesting a role as an adaptor/accessory adaptor (Salazar, Craige et al. 2005, Borner, Harbour et al. 2006, Salazar, Craige et al. 2006, Salazar, Zlatic et al. 2009). Does BLOC-1 deficiency affect cargo content of target organelles? In brain BLOC-1 is required for the targeting of synaptic vesicle proteins to the nerve terminal (Newell-Litwa, Salazar et al. 2009, Newell-Litwa, Chintala et al. 2010). For example, BLOC-1 regulates the synaptic targeting of the AP-3 cargo phosphatidylinositol-4-kinase type II alpha (Larimore unpublished results and (Craige, Salazar et al. 2008, Salazar, Zlatic et al. 2009)). This kinase is targeted to presynaptic and postsynaptic compartments (Larimore unpublished results). Thus, it could be speculated that a downstream consequence of reduced levels of dysbindin in

schizophrenia brain may be related to either defective targeting of synaptic vesicle proteins or presynaptic receptors, such as DRD2, to or from nerve terminals of affected individuals (Levey, Hersch et al. 1993, Sesack, Aoki et al. 1994, Mengual and Pickel 2002, Wang and Pickel 2002, Bamford, Zhang et al. 2004, De Mei, Ramos et al. 2009, Howes and Kapur 2009, Perreault, Hasbi et al. 2010) as well as defective targeting to or from postsynaptic compartments (Tang, Yang et al. 2009).

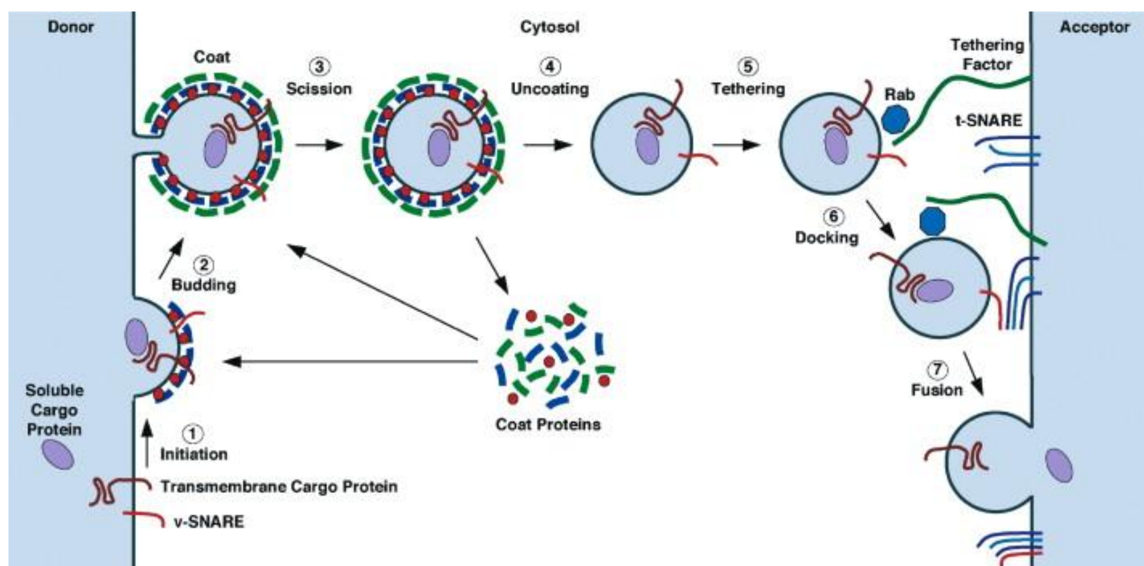


Figure 5. The stages of vesicle budding and fusion. 1) Iniation of budding begins with coat assembly at the donor membrane. Coat components (shown in blue) are recruited the donor membrane by GTPases (red) or membrane lipids. Cargo and SNAREs to be incorporated in budding vesicles cluster at the site of coat assembly. 2) Budding occurs as the more distal coat components (green) are recruited. Cargo is concentrated and donor membrane curvature increases. 3) Scission occurs when the neck of the budding vesicle is severed from the donor compartment. 4) Following budding, the vesicle undergoes Uncoating in part due to GTPase inactivation and phosphoinositide hydrolysis. Cytosolic coat proteins are released from the vesicle and recycled for future budding events. 5) Removal of coat proteins leaves the vesicle "naked", allowing it to move to the acceptor compartment membrane, likely guided through interactions with the cytoskeleton, where it can interact with Rab tethering factor to engage in docking. 6) Docking occurs as the v- and t- SNAREs form the tetrahelical bundle, or the "*trans*-SNARE complex", which allows for. (7) Fusion (Bonifacino and Glick 2004).

Additionally, BLOC-1 null *muted* or *pallid* brains possess reduced levels of the synaptic vesicle protein SNARE VAMP7/TI-VAMP (Newell-Litwa, Chintala et al. 2010). These phenotypes correlate with changes in the content of VAMP7/TI-VAMP in synaptic vesicles (Newell-Litwa, Salazar et al. 2009). Similarly, BLOC-1 loss-of-function affects targeting of cargoes in other cells derived from the neural crest like melanocytes. *Muted* BLOC-1-null melanocytes possess defective targeting of the Menke's copper transporter to melanosomes, a targeting event independent of AP-3 (Setty, Tenza et al. 2008). Collectively, although circumstantial, these results argue for a role of BLOC-1 as an adaptor/accessory adaptor that either operates in an AP-3 dependent or independent manner.

Membrane Fusion-Secretion

Membrane fusion is specified by successive mechanisms, which involve rab GTPases, single- or multi-subunit tethers, SM (Sec1/Munc18-like) proteins, and SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) (Jahn and Scheller 2006, Wickner and Schekman 2008, Sudhof and Rothman 2009, Brocker, Engelbrecht-Vandre et al. 2010). SNAREs are necessary and sufficient to mediate membrane fusion. However, eukaryotic cells posses tethering mechanisms to bring in close proximity membranes before SNARES-mediated fusion takes place. Multisubunit tethering complexes in target membranes bind SNAREs and they recognize rab GTPases present in the incoming vesicle (Brocker, Engelbrecht-Vandre et al. 2010). The interaction between the tether and the GTPase brings vesicle and target in close proximity for fusion. Downstream of tethering, SNARE residing in a vesicle (v- or R-

SNARE) and a target membrane (t- or Q-SNARE) ‘zipper’ from their N-terminal ends towards their C-terminal membrane anchoring domains, forming a tetrahelical parallel bundle. SNARE zippering brings membranes in close apposition to initiate fusion (see below). The prevalent model is that SNAREs directly function as fusion catalysts (Jahn and Scheller 2006, Wickner and Schekman 2008). Once fusion occurs the resulting cis SNARE tetrahelical parallel complex is resolved into individual SNARES, which are targeted back to their resident membranes. (Jahn and Scheller 2006, Wickner and Schekman 2008, Brocker, Engelbrecht-Vandre et al. 2010). BLOC-1 likely participates in multiple stages of this elaborated sequence of events. In fact, BLOC-1 subunits either biochemically or genetically interact with key molecules required at different stages of fusion which include rabs (Cheli, Daniels et al. 2010), SNAREs (Huang, Kuo et al. 1999, Lu, Cai et al. 2009, Ghiani, Starcevic et al. 2010), SM proteins (Hikita, Taya et al. 2009), and subunits of tethers such as the exocyst (Bao, Lopez et al. 2008, Rodriguez-Fernandez and Dell'Angelica 2009, Mead, Kuzyk et al. 2010). However, the precise mechanism and the subcellular sites of action of BLOC-1 in fusion remain to be explored in detail. In this section we will summarize the known molecular associations and functional studies implicating BLOC-1 subunits in membrane fusion. In Chapter III, I will provide further genetic evidence in support of BLOC-1 in synaptic vesicle fusion, as well as describe a possible mechanism by which interactions between fusion machinery and BLOC-1 regulate synaptic activity.

There are already several studies describing BLOC-1 biochemical interactions with various SNAREs. The BLOC-1 subunit pallidin interacts with the early endosomal SNARE syntaxin13, whereas the BLOC-1 snapin with the late endosome SNARE syntaxin 8, and the plasma membrane t-SNAREs SNAP23-25, respectively (Huang, Kuo et al. 1999, Ilardi, Mochida et al. 1999, Buxton, Zhang et al. 2003, Lu, Cai et al. 2009, Ghiani, Starcevic et al. 2010). BLOC-1 deficiencies alter the distribution of SNARES and/or decrease the cellular content of SNAREs (Salazar, Craige et al. 2006, Lu, Cai et al. 2009). Also, BLOC-1 *muted* null-mice posses altered content of the lysosomal/synaptic vesicle SNARE VAMP7/TI-VAMP in synaptic vesicles (Newell-Litwa, Salazar et al. 2009, Newell-Litwa, Chintala et al. 2010). Furthermore, over expression of the BLOC-1 subunit dysbindin increases the expression of SNAP25, a phenotype that correlates with increased basal and induced glutamate secretion in cultured neurons (Lu, Cai et al. 2009). In Chapter III of this dissertation, I genetically and biochemically demonstrate an interaction of BLOC-1 with the central components of the vesicle fusion machinery, namely N-Ethylmaleimide Sensitive Factor (NSF) and SNAREs. My work defines N-Ethylmaleimide Sensitive Factor (NSF) as a novel BLOC-1 interactor capable of modifying specialized synaptic plasticity in the fly and whose cellular content is sensitive to BLOC-1 down-regulation. The best evidence implicating BLOC-1 subunits in secretion/fusion comes from the analysis of regulated secretion in snapin and dysbindin null mice. Snapin regulates association of the putative Ca^{2+}-sensor synaptotagmin with the synaptic SNARE complex and the absence of snapin decreases calcium-regulated exocytosis of chromaffin granules (Ilardi, Mochida et al. 1999, Tian, Wu et al.

2005). In addition, snapin null neurons have reduced frequency of miniature excitatory postsynaptic events, smaller release-ready vesicle pool size, and desynchronized synaptic vesicle fusion (Pan, Tian et al. 2009). Similarly, dysbindin-null mice are characterized by larger vesicle size, both in synaptic vesicles from hippocampal neurons and chromaffin granules from adrenomedulla cells, slower quantal vesicle release, a reduced frequency of miniature excitatory postsynaptic events, and smaller total population of the readily releasable vesicle pool (Chen, Feng et al. 2008). These findings may account for in vivo microdialysis results of dysbindin-null mice, which reveal a decreased depolarization-induced dopamine release in the in the prefrontal cortex (Nagai, Kitahara et al. 2010). Although, neither of these studies has interpreted their results as derived from deficiencies in the whole BLOC-1 complex, the similarity of these secretory phenotypes in snapin- and dysbindin-null neuronal/neuroendocrine cells argues in favor of a BLOC-1 complex-dependent phenotype rather than phenotypes emerging from a single subunit deficiency. The convergence of BLOC-1 subunits in regulation of synaptic functions will be addressed in Chapter II of this dissertation. A recent and exciting development is the finding in *Drosophila* that dysbindin participates presynaptically in adaptive, homeostatic modulation of vesicle release modulating the calcium dependency of vesicle release (Dickman and Davis 2009). These findings are exciting as they suggest that specialized synaptic vesicle pools or variations in the coupling of calcium sensors and the fusion machinery may be linked to dysbindin and possibly the BLOC-1 complex. My dissertation research focuses on these predictions. In Chapter II, I will explore

the hypothesis that specialized synaptic vesicle pools are governed by BLOC-1. Next, in Chapter III, I will provide evidence to support the hypothesis that dysbindin-BLOC-1 regulates this homeostatic phenotype through interactions with the fusion machinery component NSF.

How can these secretory phenotypes be interpreted? A pressing question is the precise mechanism(s) by which the absence of BLOC-1 subunits, snapin and dysbindin, triggers secretory phenotypes. In the case of snapin, the coupling with the calcium sensor synaptotagmin 1 is a strongly supported mechanism. However, secretory phenotypes could be additionally interpreted as emerging from the capacity of BLOC-1 to bind individual SNAREs as part of a sorting mechanism. Thus, defective concentration of individual SNAREs into vesicles or target membranes could account for secretory phenotypes. Alternatively, BLOC-1 could bind to SNARE tetrahelical complexes. BLOC-1 binds to SNAP25 in vitro yet when this SNARE is presented in a tetrahelical SNARE complex, binding of BLOC-1 is impaired (Ghiani, Starcevic et al. 2010). However, there is low level of BLOC-1 association to the tetrahelical complex (Ghiani, Starcevic et al. 2010). This suggests that BLOC-1 could be involved either in regulating assembly, clamping of trans-SNARE tetrahelical complexes, or affecting either the targeting or resolution of cis-SNARE tetrahelical complexes. These proposed BLOC-1 mechanisms could operate in isolation or in concert. Data presented in Chapter III illustrate that BLOC-1 interacts with NSF mainly in the absence of SNAREs, supporting a model where the binding of monomeric SNAREs to BLOC-1 is resolved by NSF. The mild character of the neuronal secretory phenotypes in

dysbindin- and snapin-null neurons as compared with core SNARE deficiencies (Schoch, Deak et al. 2001, Washbourne, Thompson et al. 2002) suggest that these BLOC-1 subunits play modulatory roles in synaptic vesicle fusion and/or affect a subpopulation of vesicles where SNAREs, other than VAMP2 and SNAP25, determine the fusion event. This prediction is at the core of my dissertation research.

Deficiencies of the BLOC-1 subunits pallidin or dysbindin modify neuronal architecture affecting process extension, growth cone morphology, or spine length (Ito, Morishita et al. , Kubota, Kumamoto et al. 2009, Ghiani, Starcevic et al. 2010). Each one of the fundamental cellular processes previously described - sorting, fusion, transcription and cytoskeletal regulation- could either by itself or in combination account for morphological phenotypes observed in dysbindin/BLOC-1 loss-of-function. As observed in Chapter II, higher order cellular, tissue, and system phenotypes are likely to result from a combination of molecular defects defined by the protein-protein interactions engaged by dysbindin. It is plausible that there will be functions of dysbindin that occur independently of the BLOC-1 complex. However, since the most evolutionarily conserved, better documented, and higher stoichiometry dysbindin interactions are those that position dysbindin within the BLOC-1 complex, we advocate for an expansion of dysbindin studies to consider deficiencies in other BLOC-1 subunits null alleles or loss-of-function approaches. This strategy has already been pioneered (Iizuka, Sei et al. 2007) to expand the repertoire of molecules to buttress suspected cellular mechanisms contributing to the genesis of

schizophrenia.

The precise understanding of the molecular interactions and cellular functions engaged by molecules implicated in schizophrenia, such as dysbindin, will shed light into mechanisms that when impaired contribute to disease pathogenesis to a certain degree. However, it is likely that multiple cellular processes in different combinations may be affected in order to trigger schizophrenia. Thus, it is imperative to expand our knowledge in multiple molecular candidates of disease susceptibility. A monochromatic focus on just a few molecules is unlikely to unravel this complex disease.

Section 3. Organization and Trafficking of Synaptic Vesicles

Precise organization and trafficking of synaptic vesicles within the presynaptic compartment is essential for proper neurotransmission. As described, synaptic vesicle precursors are generated from an endosomal compartment in the cell body, and then trafficked down the axon to the nerve terminal. Within the presynaptic compartment, vesicles are segregated into distinct functional pools- the readily releasable pool (RRP) and the reserve pool (RP) - based on release properties (Rizzoli and Betz 2005, Gaffield, Rizzoli et al. 2006, Denker and Rizzoli 2010, Hoopmann, Punge et al. 2010, Denker, Bethani et al. 2011) that I will describe shortly. The progression of vesicles within these pools is not well understood; however, the identity and movement of vesicles into these pools is believed to be determined by distinct membrane protein composition and SNARE-dependent targeting (Broadie 1995, Schoch, Deak et al. 2001, Rizzoli and Betz 2005, Bethani, Lang et al. 2007, Welzel, Henkel et al. 2011, Raingo, Khvotchev et al. 2012).

Within the RRP, vesicles are loaded with the necessary neurotransmitter (Katz, Chase et al. 1969) and prepared for fusion through a series of steps that lead to SNARE complex formation and opening of the fusion pore (Südhof 2013). The essential step in vesicle fusion is the binding of the SNARE protein residing on the vesicle (v/R-SNARE) to the SNAREs residing on the target membrane (t/Q-SNAREs). Synaptic vesicles in the RRP contain the v-SNARE synaptobrevin (also called VAMP2), as well as the calcium-sensing protein synaptotagmin, and bind the plasma membrane localized t-SNAREs syntaxin 1 and SNAP-25 (Söllner, Bennett et al. 1993, Monck and Fernandez 1994). Conformational changes in

syntaxin-1 allow for the initiation of SNARE complex formation and vesicle docking within the active zone of the presynaptic membrane (Broadie, Prokop et al. 1995, Dulubova, Sugita et al. 1999, Misura, Scheller et al. 2001). SNARE complex formation proceeds in a 'zippering' process to bring the membranes into close apposition, as described above (Figure 6, Priming I) (Otto, Hanson et al. 1997). Partially assembled SNARE complexes bind complexins, which primes SNARE complexes for synaptotagmin association (Reim, Mansour et al. 2001, Giraudo, Eng et al. 2006, Tang, Maximov et al. 2006, Melia 2007). Complexin-primed complexes can then associate with synaptotagmin and thus, bind calcium (Ca^{2+}) (Reim, Mansour et al. 2001, Cai, Reim et al. 2008, Jorquera, Huntwork-Rodriguez et al. 2012), which enters the cell through nearby voltage-gated calcium channels and triggers the opening of the fusion pore (Figure 6) (Katz and Miledi 1969, Baker, Hodgkin et al. 1971, Kaeser, Deng et al. 2011, Eggermann, Bucurenciu et al. 2012).

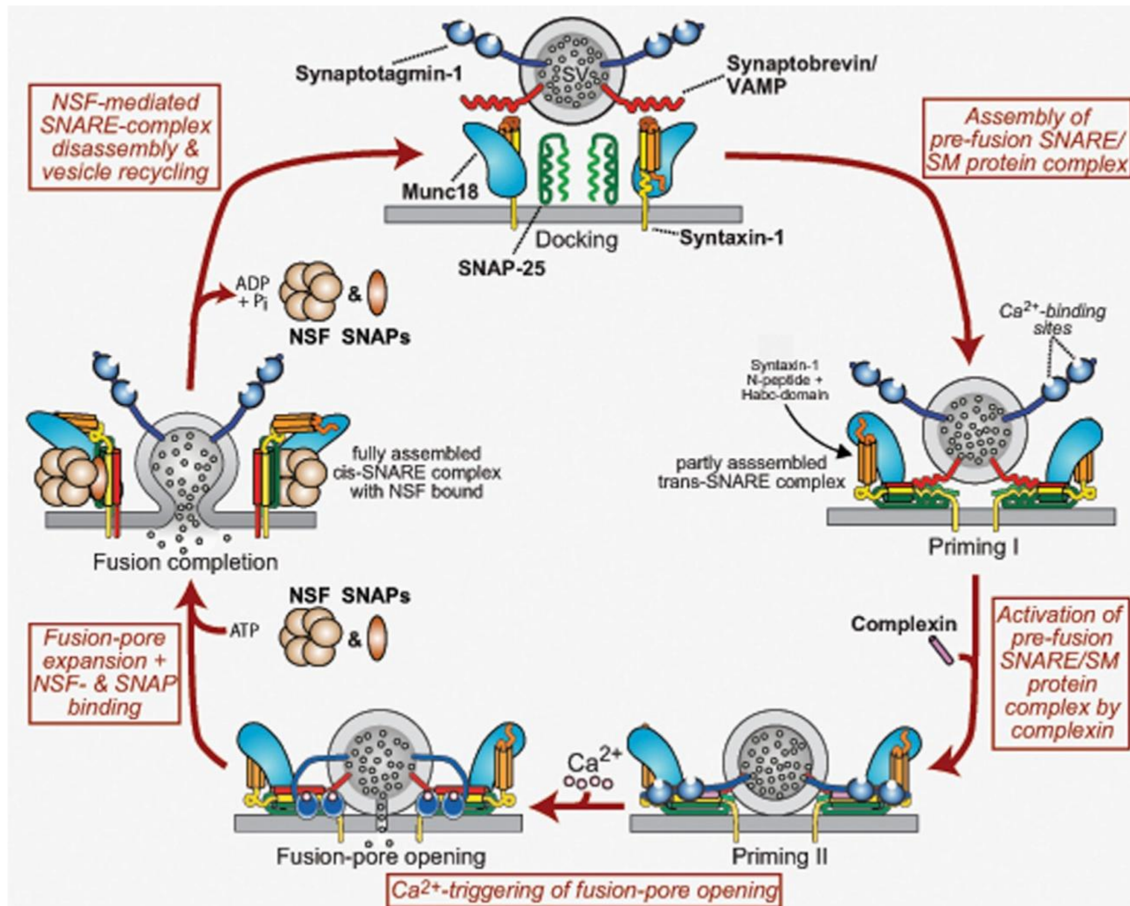


Figure 6. Synaptic vesicle fusion cycle. Fusion is initiated by docking of the synaptic vesicle and through zippering of the v-SNARE synaptobrevin with the t-SNARE syntaxin-1. Trans-SNARE complex formation proceeds through priming steps involving SM protein Munc18 binding (Priming I), followed by complexin binding and synaptotagmin association with the the cis-SNARE complex (priming II). Calcium entry through voltage-gated ion channels and subsequent binding to synaptotagmin triggers fusion-pore opening. SNAP and NSF recruitment immediately following fusion resolve cis-SNARE complex in an ATP-dependent manner. NSF-mediated SNARE complex disassembly allows for clathrin mediated endocytosis and vesicle recycling (Südhof 2013).

A concerted set of steps also regulates vesicle endocytosis. In the absence of stimulation, individual vesicles undergo spontaneous fusion with the plasma membrane of the active zone. Vesicle fusion at the plasma membrane recruits alpha-SNAP, which binds to SNAREs in the cis/SNAREpin conformation. Alpha-SNAP, in turn, recruits the AAA ATPase NSF (*N*-ethylmaleimide-sensitive factor), which unwinds the SNARE-pin in an ATP-dependent fashion (Figure 6) (Söllner, Bennett et al. 1993, Monck and Fernandez 1994, McMahon and Südhof 1995, Mayer, Wickner et al. 1996). Following spontaneous vesicle fusion events as well as baseline synaptic transmission, membrane at the site of fusion is rapidly retrieved by clathrin-mediated and AP-2 dependent endocytosis to an early endosome in order to maintain the readily releasable vesicle pool as the overall integrity of the presynaptic plasma membrane (Heuser and Reese 1973, Ullrich, Li et al. 1994, Jarousse and Kelly 2001, Wu et al. 2007, Haucke et al. 2011). Here, synaptic membrane proteins are sorted into vesicles to replenish RRP at the active zone (Figure 7). At this time, there is little to no mixing of the reserve pool with recycling vesicles (Opazo, Punge et al. 2010). During periods of high frequency (10 Hz) stimulation, however, the readily releasable pool and clathrin-mediated endocytosis are not sufficient to support the high demand for synaptic vesicle release and recycling. In these instances, vesicles are mobilized from the reserve pool to sustain prolonged periods of excitation and high-frequency stimulation (Gaffield, Rizzoli et al. 2006, Denker, Kröhnert et al. 2009, Denker and Rizzoli 2010, Denker, Bethani et al. 2011). Additionally, repopulation of synaptic vesicles by clathrin-dynamin dependent mechanisms and endosomal sorting is supplemented by a process known as activity-dependent bulk

endocytosis (Clayton and Cousin 2009, Cousin 2009, Wu, O'Toole et al. 2014). Through this process, deep invaginations form from the plasma membrane, which can either bud synaptic vesicles directly, or can give rise to endosomal intermediates that then sort membrane proteins into the appropriate synaptic vesicle pools (Figure 7) (Heuser and Reese 1973, Clayton and Cousin 2009, Cousin 2009, Wu, O'Toole et al. 2014). As described in Section 2, Arf1 in its GTP conformation recruits the clathrin adaptors AP-1 and AP-3 to endosomal intermediates to facilitate budding (Ooi, Dell'Angelica et al. 1998, Robinson 2004, Newell-Litwa, Seong et al. 2007, Dell'Angelica 2009). A subset of synaptic vesicles budding from the endosome are then targeted to the reserve pool in an AP-3/BLOC-1 dependent process (Figure 7) (Grabner, Price et al. 2006, Newell-Litwa, Salazar et al. 2009, Asensio, Sirkis et al. 2010).

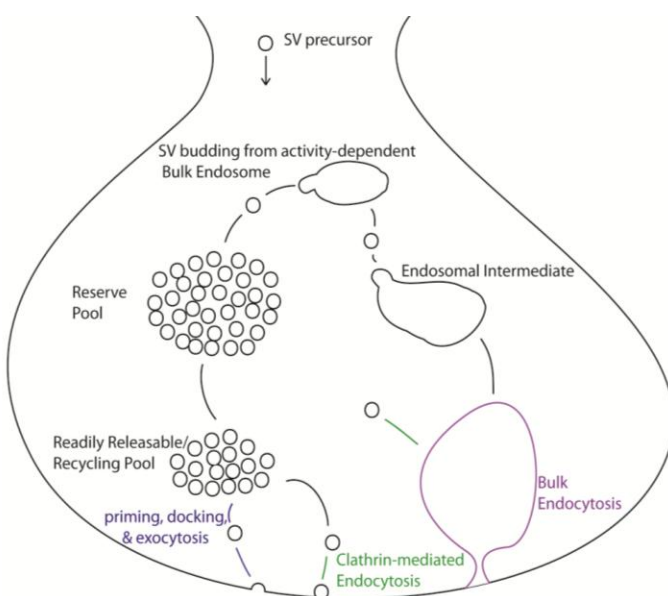


Figure 7. Synaptic vesicle pool organization and endocytic pathways. Within the presynaptic compartment, synaptic vesicles are segregated into two functionally distinct pools- reserve pool and readily releasable pool (RRP). The readily releasable pool of vesicles contains vesicles competent for immediate fusion, which then recycle between the plasma membrane and the RRP via clathrin-meidated and AP-2 dependent endocytosis, through an early endosome compartment (not shown for simplicity). The reserve pool is recruited during periods of sustained high-frequency stimulation and is replenished through vesicle budding following activity-dependent bulk endocytosis rather than clathrin-mediated mechanisms.

Section 4. *Drosophila melanogaster* as a model system.

Why choose to study BLOC-1 regulation of synaptic neurotransmission in *Drosophila melanogaster*? The fruit fly presents a unique opportunity to address the questions I have raised in regards to the effects of complex genetics on neurotransmission in neurodevelopmental and psychiatric disorders that cannot be addressed in rodent models or cells in culture. While we cannot expect to perfectly model any complex neurological disease, such as schizophrenia, in the fruit fly, it does allow us to test for common endophenotypes in manipulated human genes or homologs as we try to unravel a convergent pathway of disease. Basic schizophrenia pathogenesis hypotheses tell us that the molecular defects concentrate in the synapse of affected individuals to modify individual synapses, and that synaptic changes give rise to an enhanced saliency to stimuli that manifests as the systems level defects responsible for positive symptoms of the disease. *Drosophila* offers a unique set of tools to assess molecular changes within the synapse and on individual synaptic function, as well as on complex, circuit-based behaviors. Through my dissertation research, I assess molecularly-governed synaptic phenotypes as well as saliency to stimuli, and address the genetic fidelity and correlation between phenotypes. Thus, although we may not be able to reconstruct the disease, we can precisely manipulate *Drosophila* homologs of genes associated with schizophrenia in humans to test their influence on both behavioral and synaptic phenotypes in response to stimulation changes and challenges. In fact, this is exactly the case when we consider the first two studies of schizophrenia-associated genes already in *Drosophila*. The first study observed changes in sleep patterns when human DISC1, which was

introduced in Section 1, was expressed in fruit flies (Millar, Wilson-Annan et al. 2000). Sleep-wake cycles are commonly disrupted in schizophrenia patients, but are not the underlying cause of the disease (Bromundt, Köster et al. 2011),(Monti and Monti 2004). This study offered proof that correlative human psychiatric disease endophenotypes (i.e. sleep disruption) could be used as read outs in *Drosophila* transgenic models. The second, which was previously described in Section 2, demonstrated that loss of function mutations in *dysbindin* block synaptic homeostasis (Dickman and Davis 2009). While the first study demonstrates that similar phenotypes may exist between human patients and fruit flies, the latter suggests that *Drosophila* can be used to identify and study an underlying link- synaptic homeostasis- in mechanisms of the disease.

This use of the fruit fly as a model system to understand neurological disorders is best illustrated by the use of *Drosophila* to study Fragile X syndrome (FXS). FXS is a neurodevelopmental disorder characterized by intellectual disability, autism spectrum disorder, stereotypies, epilepsy and dismorphia. FXS is caused by loss of function mutations in the Fragile X Mental Retardation 1 gene (*FMR1*) in humans. *FMR1* encodes an RNA binding protein that regulates translation of many proteins mostly as a transcriptional repressor (Krueger and Bear 2011),(Oostra and Verkerk 1992). The *FMR1* product, fmr1, is highly expressed in the central nervous system (Devys, Lutz et al. 1993). Unlike schizophrenia, FXS patients have a stereotyped set of behavioral and physical phenotypes in addition to mental retardation (Laxova 1994), many of which are recapitulated in *FMR1* knockout mice, such as hyperactivity, sleep disorders, and even enlarged testes

(1994). However, until the homologous *Drosophila FMR1* gene (*dfmr1*) was identified and characterized as a highly conserved homolog of the mammalian gene (Wan, Dockendorff et al. 2000), little was known about the downstream targets of fmr1 and how these targets led to the dramatic phenotypes. Following identification of *dfmr1*, it was found that absence of the encoded protein dFMR1 in *Drosophila* leads to increased branching and dendritic arbour complexity in the larval NMJ, while overexpression of dFMR1 has the exact opposite effect (Zhang, Bailey et al. 2001). Additionally, it was found that dFMR1 null adults have defects in maintaining circadian rhythms and sleep patterns (Dockendorff, Su et al. 2002, Inoue, Shimoda et al. 2002, Morales, Hiesinger et al. 2002), have short- and long-term memory (McBride, Choi et al. 2005),(Bolduc, Bell et al. 2008, Banerjee, Schoenfeld et al. 2010), display locomotor hyperactivity (Inoue, Shimoda et al. 2002), and have enlarged testes (Zhang, Matthies et al. 2004). If phenotypes in mutant flies so precisely replicate symptoms in humans, could response to treatment in flies also accurately predict response to treatment in humans? This predictability of and consistency of phenotypes from *Drosophila* to mice to humans presented a unique opportunity for the rapid screening and development of novel pharmacological interventions for treatment of FXS. It was quickly found that treatment of dFMR1 mutants with mGluR antagonists reversed the morphological and behavioral effects in fruit flies (McBride, Choi et al. 2005), and mGluR5 antagonists are now in clinical trials for use in humans (Jacquemont, Curie et al. 2011),(Berry-Kravis, Hessl et al. 2009).

Fragile X Syndrome and schizophrenia are very different genetic disorders, with FXS being a strictly monogenic disorder that gives rise to a consistent set of symptoms across patients, while schizophrenia likely arises from the loss of and interaction between many genes which gives rise to a greater spectrum of phenotypes. However, the successful use of endophenotypes in *Drosophila* to study FXS, and the subsequent identification of a means of pharmacological intervention, lays the groundwork for the use of the fruit fly as a legitimate model system to facilitate future understanding and treatment of more complex neurological disorders.

Drosophila as a model system has been so successful in garnering information towards our understanding of FXS largely due to the close homology between *fmr1* and *dfmr1 (O'Kane 2011)*. Importantly for the present studies, orthologous *Drosophila* genes encoding homologs to all eight of the mammalian BLOC-1 subunits have been recently identified (Table 1) (Cheli, Daniels et al. 2010). While these gene products had been identified in the fruit fly, the functions and interactions of the encoded proteins remained largely unexplored at the time of this study.

Table 1. Homologues of human BLOC-1 subunits encoded by the fruit fly genome

Human subunit	*Drosophila* gene Current name	Proposed name	*E*-value[a]	Amino acid identity[b] (%)
BLOS1	*CG30077*	*blos1*	6×10^{-31} (1)	55
BLOS2	*CG14145*	*blos2*	7×10^{-16} (1)	40
BLOS3	*CG34255*	*blos3*	9×10^{-6} (2)	22
Cappuccino	*CG14149*	*blos4*	2×10^{-4} (1)	25
Dysbindin	*CG6856*	*dysbindin*	6×10^{-15} (1)	38
Muted	*CG34131*	*muted*	2×10^{-7} (2)	18
Pallidin	*CG14133*	*pallidin*	1×10^{-3} (1)	24
Snapin	*snapin*	*snapin*	3×10^{-13} (1)	33

In Chapter II, I will focus on the role of genetic interactions between BLOC-1 subunits on synaptic neurotransmission at the *Drosophila* neuromuscular junction (NMJ). The *Drosophila* NMJ has been extensively studied as a model synapse for many decades (Bellen, Tong et al. 2010). In invertebrates, synaptic transmission at the NMJ is mediated by neurotransmitter glutamate, while acetylcholine is prevalent in the invertebrate central nervous system (CNS). In contrast, glutamate acts as the primary excitatory neurotransmitter within the vertebrate CNS, while acetylcholine is the neurotransmitter of choice at the vertebrate neuromuscular junction (Bellen, Tong et al. 2010, O'Kane 2011). Synaptic structures at the NMJ contain homologs to fusion machinery components, ion channels, and postsynaptic receptors found in the mammalian CNS, which contribute to the appeal of the *Drosophila* NMJ as a model synapse (Broadie 1995). Further, it has been well studied and is easily accessible for examination by both electrophysiological and immunohistochemical techniques, making it easy to identify defects in neurotransmission and morphology (Jan and Jan 1976, Südhof, De Camilli et al. 1993). In these studies, I will focus on muscle 6 in abdominal segments 2 and 3 of the third instar larvae, which is among the most well characterized of model synapses (Figure 8).

Additionally, the fruit fly possesses a simple, three chromosome (plus Y) genome, which is easily amenable to manipulation and thus, genetic studies. In particular, the GAL4/UAS system is a powerful genetic tool that allows for targeted gene expression or gene expression down regulation in the fruit fly (Duffy 2002).

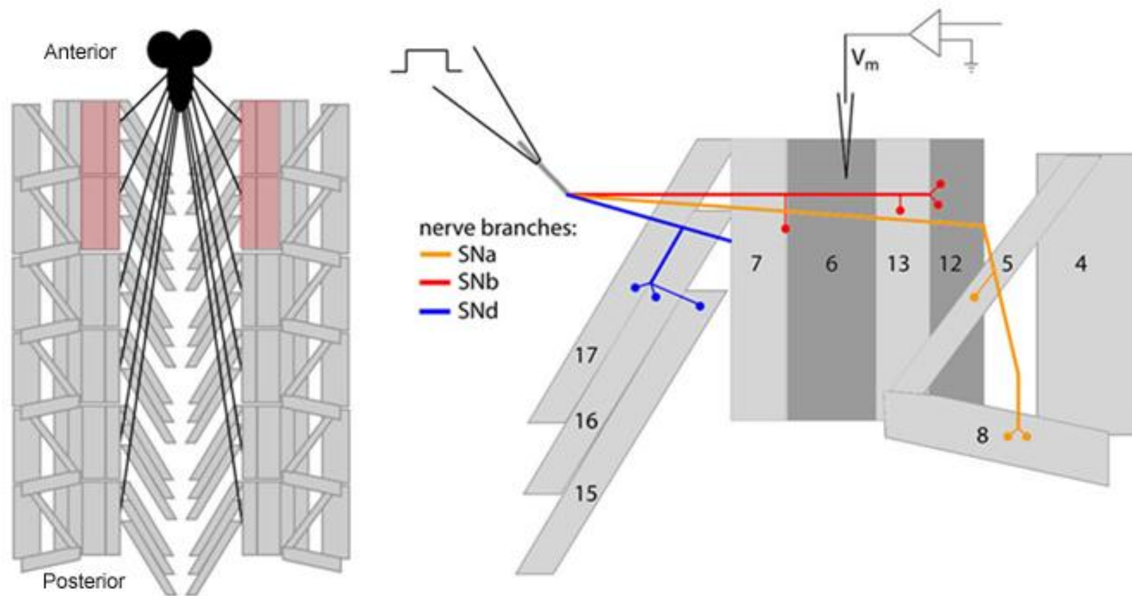


Figure 8. Schematic of the *Drosophila* neuromuscular junction. At left, the musculature of the *Drosophila* third instar larva is shown in grey. Muscle organization is repeated in segments down the length of the animal. Red boxes highlight muscles 6 and 7 in abdominal segments A2 and A3. The central ganglion at the anterior of the animal is shown in black with emmanating nerves shown innervating each muscle segment. At right, an enlargement of the grey muscles showing electrophysiological experimental paradigm. Severed motor neurons (in blue, orange, and red) are taken into a glass stimulating electrode (upper left), and a pulse is delivered. Changes in membrane voltage is recorded at muscle 6.

GAL4 is a yeast transcription factor that regulates transcription of a number of yeast genes by binding to DNA sequences adjacent to the open reading frame (ORF) of genes called Upstream Activating Sequence (UAS) sites (Giniger, Varnum et al. 1985, Giniger and Ptashne 1988, Duffy 2002). In *Drosophila*, however, the GAL4 sequence (or 'driver') is engineered separate from the UAS site. Further, the UAS site is engineered with the responder gene of interest downstream (Fischer, Giniger et al. 1988, Brand and Perrimon 1993, Duffy 2002). Using this bipartite approach, the responder gene is under the control of the UAS element, which requires the presence of GAL4 for its own transcription.

Thus, in the absence of GAL4, the responder gene is transcriptionally silent. Additionally, by placing GAL4 transcription under the control of tissue-specific promotors, GAL4 expression can be controlled in time and space, restricted to a specific tissue or set of cells within a tissue, or restricted within a developmental window (Brand and Perrimon 1993, Duffy 2002). Thus, when flies carrying the responder gene are crossed to flies expressing GAL4, the resultant progeny will express the responder gene in a transcriptional pattern consistent with GAL4 expression (Figure 9). Conversely, GAL4 expression can also be suppressed through the use of the GAL4 inhibitor GAL80, which binds GAL4 and prevents transcriptional activation (Lee and Luo 1999).

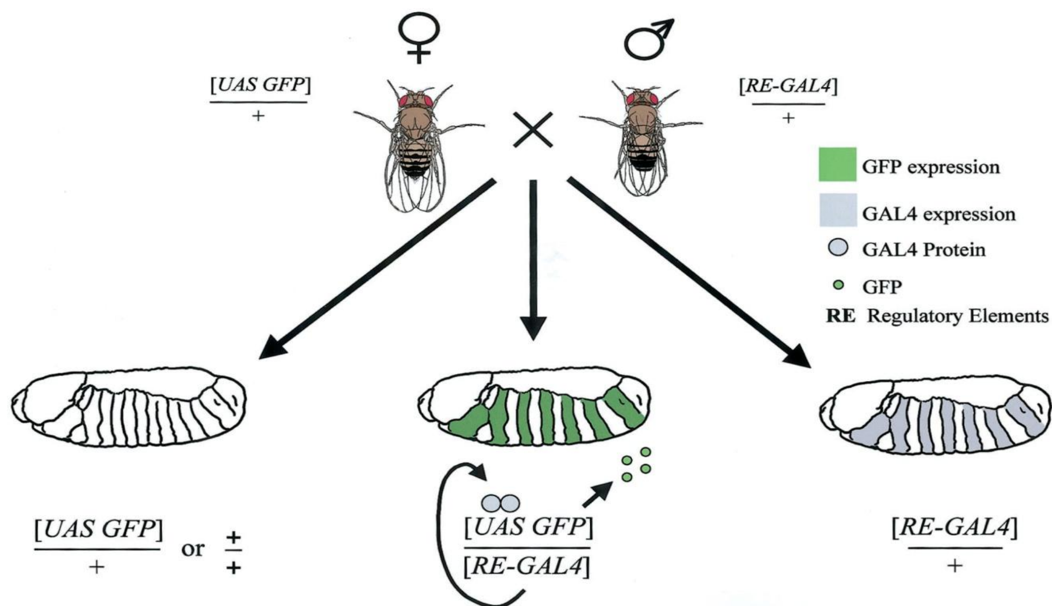


Figure 9. GAL4/UAS system for targeted gene expression in *Drosophila*. Animals encoding the transgene containing the upstream activating sequence (UAS) and the target gene of interest (here, GFP) show wild-type pattern of expression (left larva). Animals expressing the GAL4 protein under tissue specific regulatory elements (RE) only express GAL4 in tissue where the RE are expressed (right larva). In animals containing both the UAS-GFP transgene and GAL4, GAL4 proteins bind the upstream activating sequence for the target gene (GFP), allowing for GFP expression in tissues which also express GAL4 (middle larva) (Duffy 2002).

Contributions of this dissertation research

My dissertation research addresses two fundamental questions that make significant contributions at the intersection of the fields of synaptic vesicle trafficking, neurological conditions, and polygenic disorders. Not only do I address fundamental questions regarding BLOC-1 assembly in a common model system, *Drosophila melanogaster*, but I also address the much broader questions of, "How do we study genetically and phenotypically complex polygenic neurological disorders in a laboratory setting?". My dissertation research focuses on BLOC-1 as an example of a protein complex, with a defined network of interacting partners, involved in synaptic vesicle trafficking and neurological disease. I address my central hypothesis that:

> **BLOC-1 interactions, both within the complex as well as with identified binding factors outside of the complex, regulate synaptic function, plasticity, and behavior.**

In Chapter II, I will discuss the implications for studying components of a tightly interacting complex of proteins in isolation. I use BLOC-1 as an example protein complex to explore the consequences of loss-of-function mutations on synaptic functions dependent on BLOC-1 trafficking. Using a multiparameter approach, I demonstrate that perturbations to a single complex member can render the remaining components unstable, and the ramifications on synaptic function are genetically unpredictable. Additionally, combinations of loss-of-function mutations do not necessarily exacerbate a given phenotype. Rather, within a defined protein complex, loss-of-function combinations can have deleterious effects, leading to gain-of-function remnants and/or partial loss-of-function of

the complex, a concept that has not yet been demonstrated in membrane-trafficking. I extend this further to show that small changes at the single synaptic level can be indicative of impairments in plasticity and behavior that may underlie disease.

In Chapter III, I extend the question of studying complex genetic disorders to looking at interactions outside of the complex. I successfully use a biochemically curated interactome and experimental endophenotypes to guide investigation of underlying disease mechanisms. Additionally, by doing so, I demonstrate that components that biochemically interact and are sensitive to the cellular content on one another, converge in a functional pathway. Having identified vesicle fusion machinery as sensitive to BLOC-1 cellular levels, and confirmed the biochemical interactions of BLOC-1 with fusion machinery components, I test the ability of the identified network component NSF to converge in a functional pathway with BLOC-1, and to rescue BLOC-1 deficits in synaptic neurotransmission.

These studies provide insight into how network stability and genetic interactions at play in neurodevelopmental disorders can lead to a wide spectrum of phenotypes. Most importantly, my research demonstrates that polygenic disorders cannot be understood through classical monogenic experiments.

CHAPTER II

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

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ABSTRACT

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 *Bloc1s1* 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 within a defined neurodevelopmental regulatory network, precise stoichiometry, rather than merely the number of alleles affected within the network, determines phenotypic outcomes.

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). Components of these networks have been identified by studying single gene disruptions, which provide fundamental insight into the necessity and sufficiency of a single gene product for a neuronal phenotype. However, the general assumption in these studies is that gene products are organized into linear pathways rather than networks, such that the remaining components remain stable following disruption of a single gene (Greenspan 2009). This model is at odds with the genetics of neurodevelopmental disorders where complex behavioral and cognitive phenotypes emerge from the simultaneous modification of a number of genes, which align along a chromosomal segment rather than a pathway (Stefansson, Ophoff et al. 2009, Bassett, Scherer et al. 2010, Malhotra and Sebat 2012, Rapoport, Giedd et al. 2012, Moreno-De-Luca, Myers et al. 2013, Stefansson, Meyer-Lindenberg et al. 2014). This raises the question of how two or more genes interact to specify simple and complex neuronal traits. We focus on this question as it has been largely unexplored, yet holds promise for our understanding of the pathogenesis of polygenic neurodevelopmental disorders.

Schizophrenia is a heritable polygenic neurodevelopmental disorder where defective synaptic mechanisms contribute to disruptions at higher levels of neuronal organization (Gottesman and Shields 1967, Mirnics, Middleton et al. 2000, Purcell, Wray et al. 2009, Faludi and Mirnics 2011, Purcell, Moran et al.

2014). Synaptic mechanisms underlying schizophrenia, however, remain obscure in large part due to the predominantly polygenic nature of this disorder. Hemizygous deletions, duplications, or inversions of submicroscopic chromosomal segments, known as copy number variations, are the most frequent genomic burden associated with schizophrenia (Stefansson, Ophoff et al. 2009, Bassett, Scherer et al. 2010, Malhotra and Sebat 2012, Rapoport, Giedd et al. 2012, Moreno-De-Luca, Myers et al. 2013, Ahn, Gotay et al. 2014, Stefansson, Meyer-Lindenberg et al. 2014). A single copy number variation can span multiple genes, thus creating collections of gene dosage imbalances. For example, single copy deletions of the chromosomal segment 22q11.2 encompass between 35 to 60 genes, and are the strongest genetic risk factor for schizophrenia (Murphy 2002, Ahn, Gotay et al. 2014, Schneider, Debbane et al. 2014). How these combined gene dosage imbalances interact to confer schizophrenia risk and affect the synapse and associated circuitry is unknown.

Here we model a two-loci genetic deficiency affecting a schizophrenia susceptibility network in the fly. This network is centered on the human gene *DTNBP1* and its *Drosophila* ortholog *dysb,* both of which encode Dysbindin. We previously defined the human Dysbindin protein-protein interaction network (interactome) (Gokhale, Larimore et al. 2012). The core of this Dysbindin network is comprised of Dysbindin and seven closely associated proteins, which form an octameric complex known as the biogenesis of lysosome-related organelles complex 1 (BLOC-1)(Ghiani and Dell'angelica 2011, Gokhale, Larimore et al. 2012, Mullin, Gokhale et al. 2013). Genetic polymorphisms in *DTNBP1*, are

among the strongest risk factors for schizophrenia, (Talbot, Ong et al. 2009, Mullin, Gokhale et al. 2011) especially with the more severe, early-onset childhood schizophrenia (Gornick, Addington et al. 2005, Fatjó-Vilas, Papiol et al. 2011). *DTNBP1* polymorphisms also influence cognitive and neuroanatomical traits in non-diseased humans either when *DTNBP1* polymorphisms are considered in isolation or, importantly, in association with other synaptic function genetic loci, such as *COMT* (Straub, Jiang et al. 2002, Van Den Bogaert, Schumacher et al. 2003, Bray, Preece et al. 2005, Luciano, Miyajima et al. 2009, Markov, Krug et al. 2009, Markov, Krug et al. 2010, Mechelli, Viding et al. 2010, Cerasa, Quattrone et al. 2011, Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011, Tognin, Viding et al. 2011, Wolf, Jackson et al. 2011, Ayalew, Le-Niculescu et al. 2012, Papaleo, Burdick et al. 2014). Further, post-mortem brain analysis revealed that eighty percent of schizophrenia brains contain reduced levels of dysbindin in neuroanatomical regions compromised in schizophrenia patients (Talbot, Eidem et al. 2004, Tang, LeGros et al. 2009, Talbot, Louneva et al. 2011). 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. These phenotypes should emulate phenotypic outputs in copy number variations associated with neurodevelopmental disorders.

In this study, we used *Drosophila* to understand the impact of fly loss-of-function mutations affecting BLOC-1 complex subunits on synaptic networks, as all eight

mammalian BLOC-1 complex subunits possess fly orthologues and, similar to mammalian genes, seem to follow a simple autosomal recessive inheritance pattern (Cheli, Daniels et al. 2010). We predicted that phenotypes associated with gene copy reductions affecting BLOC-1 subunits should follow a recessive inheritance pattern, and that this pattern should be congruent across synaptic mechanisms that progressively scale up in complexity. We tested the effects of four BLOC-1 complex loss-of-function genotypes on neurotransmitter release, synapse morphology, homeostatic plasticity, and behavioral/olfactory habituation. Contrary to our prediction, we found that homozygous loss-of-function alleles of BLOC-1 complex subunit genes *dysb* or *Blos1,* or compound heterozygotes in the two genes, affected synaptic functions with divergent inheritance patterns rather that the predicted simple recessive pattern. We conclude that single synapse and circuit-based phenotypes associated with a single gene dosage imbalance are non-congruently modified by a second locus encoding a protein-protein interaction network. We propose that genetic control of the stoichiometry of a neurodevelopmental regulatory network diversifies its phenotypic output in normal and disease states.

RESULTS

BLOC-1 assembles into an octameric complex in *Drosophila* neurons.

Dysbindin exists as a member of the octameric biogenesis of lysosome-related organelles complex 1 (BLOC-1) in mammalian cells (Fig. 1A). While all eight mammalian BLOC-1 subunits have *Drosophila* orthologues, the existence of this complex in *Drosophila* has not yet been established, but has been predicted from yeast two-hybrid and proteomic studies (Cheli, Daniels et al. 2010, Guruharsha, Rual et al. 2011). However, these studies only document a subset of the predicted interactions among the eight *Drosophila* BLOC-1 subunit orthologues (Fig 1B). Thus, we first set out to determine if an orthologous Dysbindin-containing BLOC-1 complex assembles in the *Drosophila* central nervous system 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 associated proteins from fly head lysates. UAS-Venus-tagged Dysbindin transgene was expressed under the pan-neuronal C155-GAL4 driver to identify neuronal proteins co-precipitating 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* orthologues in comparison to the C155 control, determined by spectral count quantitation (Fig. 1E). Proteins non-selectively bound to GFP beads, such as tubulin and elongation factor 1 alpha, were similarly represented in spectral counts in both control and Venus-Dysbindin precipitated samples (Fig. 1E). The identity of the *Drosophila* BLOC-1 subunit orthologues was confirmed by MS/MS peptide sequencing (Fig. 1F). We verified the Pallidin-Dysbindin interaction by immunoprecipitation of Venus-Dysbindin and specific detection of co-precipitating 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 incorporates into BLOC-1 in *Drosophila* neurons, and 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 NMJ.

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, Kuo et al. 1999, Zhang, Li et al. 2002, Ciciotte, Gwynn et al. 2003, Li, Zhang et al. 2003, Gwynn, Martina et al. 2004, Starcevic and Dell'Angelica 2004). We analyzed whether gene-dosage reductions in BLOC-1 subunits had the capacity to produce recessive synaptic phenotypes. We utilized 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 synapse (NMJ) 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 orthologues (Dickman and Davis 2009, Dickman, Tong et al. 2012). We assessed animals for baseline miniature excitatory junctional potential (mEJP) amplitude and frequency, evoked excitatory junctional potential (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 Fig 2A-E). Similarly, the different BLOC-1 loss-of-function alleles had no effect on the frequency of mEJPs (Fig. 2F). Consistent with previous reports, the amplitude of the mEJP was not altered in $dysb^{1}$ dysbindin mutant synapses as compared with two control strains, Canton-S or w1118 (Fig. 2G). In contrast, we found that animals carrying any of three *Blos1* null alleles, $Blos1^{EY}$ (Fig2A, insert), $Blos1^{ex2}$ and $Blos1^{ex65}$ had increased mEJP amplitudes and correspondingly lower quantal content compared to both wild type strains (Fig. 2H, L and P, respectively. Red traces). While this increase in mEJP amplitude is in agreement with the initial characterization of $Blos1^{ex2}$, we observed that this phenotype was consistent across all three null alleles (Cheli, Daniels 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 and P, respectively. Blue traces). Presynaptic expression of *Blos1* ($elav^{C155}$-Gal4; UAS-Blos1) was sufficient to

rescue the observed changes in mEJP amplitude and quantal content (Fig 2C, K and 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 as compared to *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 (*$dysb^{1}$*) and the deletion *Df (BSC 416)*, while entirely deletes the *dysb* gene as well as about 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 *$dysb^{1}$* 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 and Q. Compare red and blue traces). We observed the same result in flies double heterozygous for *Df (BSC 416)* and the *$Blos1^{EY}$* null mutation (Fig. 2J, compare red and blue traces). As expected, *Blos1* expressed in trans with the *$dysb^{RV}$*, which is a perfect excision of the *$dysb^{1}$* 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 (*elav*^{C155}-Gal4; UAS-dysb) in the *Blos1*$^{EY/+}$;*dysb*$^{1/+}$ 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). Thus, these data demonstrate that BLOC-1 subunits participate in a common presynaptic molecular and genetic network to regulate baseline neurotransmission at the *Drosophila* NMJ. Additionally, these data show that neurotransmission is sensitive to the genetic 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, Wall et al. 2004, Choi, Imlach 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, Bassetto et al. 2005, Gatto and Broadie 2008, Zhao, Wang 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 in order to quantify the number of boutons per unit muscle area (Fig. 3). We determined that *Blos1*

null alleles have significantly increased number of boutons per muscle at the 6/7 synapse (Fig. 3J and compare A with B-C). This increase in bouton number occurred without changes in muscle size across genotypes (Fig. 3K). The increased number of boutons was a dominant phenotype as it was also observed in heterozygous *Blos1* mutants (Fig. 3 compare A with D-E. Fig. 3J), a genotype-to-phenotype correlation matching the changes in mEJP amplitude and quantal content. Importantly, when a single copy loss of *Blos1* null alleles was expressed in trans with either *dysb*^{1} or the *dysb* deficiency, the bouton count phenotype was suppressed to resemble wild type numbers (Fig. 3J and compare D-E with F-G). The specificity of the suppression achieved with the *dysb*^{1} allele was tested by expressing *Blos1* mutants in trans with the *dysb*^{1} revertant (*dysb*^{rv}), a genotype that phenocopied the *Blos1* heterozygotic animals (Fig. 3J and compare F-G and H-I). These results demonstrate that there is a one-to-one correspondence between genotypes and the bouton morphology and spontaneous release phenotypes at the NMJ. In both instances *Blos1* loss-of-function alleles dominantly affect the phenotypes are suppressible by loss-of-function *dysb* allele in trans. These results indicate that similar BLOC-1 dependent genetic and molecular mechanisms control spontaneous release and synapse morphology.

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 if this genotype-to-phenotype congruency 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, Maureira et al. 2000, Dickman and Davis 2009, Frank, Wang et al. 2013). We focused our efforts on the ability for the transheterozygotic *dysb* mutations to preclude the dominant effects of *Blos1* as it suggests that synaptic mechanisms require precise BLOC-1 subunit stoichiometry.

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-minute 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. 4 A-A1 compare black and blue traces) (Dickman and Davis 2009, Dickman, Tong et al. 2012, Davis 2013, Frank, Wang et al. 2013, Frank 2014). This synaptic compensation occurs by doubling their quantal content of neurotransmitter

release over baseline (Fig. 4H). However, *dysb*^{1} mutants failed to display such presynaptic homeostatic compensation in response to this toxin, as reflected by the unchanged quantal content after toxin incubation (Fig. 4 B compare black and blue traces and Fig. 4H). Importantly, single copy loss of dysbindin does not block synaptic homeostasis (Fig. 4F and H). Unlike what we observed with the mEJP and branching dominant phenotypes, *Blos1* null (Fig. 4C and H) and heterozygous animals (Fig. 4D and H) have no defect in synaptic compensation. However, transheterozygotic *Blos1*^{EY}*;dysb*^{1} mutants blocked synaptic homeostasis to the same extent as *dysb*^{1} homozygotic mutants (Fig. 4 compare B and E and Fig. 4H). These results demonstrate that the BLOC-1 complex is necessary for synaptic homeostatic 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. 5A-C), engages the recycling pool of vesicles to maintain neurotransmission; however, intense, high frequency stimulation (10 Hz, Fig. 5D-E) mobilizes the reserve pool of vesicles to support neurotransmission(Delgado, Maureira et al. 2000, Kim, Kumar et al. 2009). None of the loss-of-function allele combinations affected the rate of vesicle depletion at low frequency stimulation (Fig. 5A-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. 5E) but not *dysb*^{1} mutants (Fig. 5A) had a significantly enhanced depletion rate of the total vesicle pool compared to wild type animals and *Blos1* single copy loss. The divergent effects of the *Blos1* and *dysb*^{1} homozygous mutants on the mobilization of the total vesicle pool did not reflect BLOC-1 independent functions of these alleles as evidenced by transheterozygotic *Blos1*^{EY}*;dysb*^{1} mutants, which phenocopied the *Blos1* homozygous animals when stimulated at 10 Hz (Fig. 5F). 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 subunits 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 GABA-ergic neurotransmission in the local interneurons (LNs) of the olfactory circuit in *Drosophila* (Das, Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013, Twick, Lee 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. 5). We explored genotype-to-phenotype relationships across different combinations of BLOC-1 loss-of-function alleles. 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-minute 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. 6A, *CS*. Compare pre and post-test in blue). In contrast, animals homozygous for the $dysb^1$ mutation showed no reduction in avoidance behavior, indicating a deficit in short-term

olfactory habituation (Fig. 6A). While the hypomorph *dysb*^{1} 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 *dysb*^{1} mutation in trans with the *dysb*^{Df}. Single-copy loss of *dysb*, either by the *dysb*^{1} or the *dysb*^{Df} mutation did not affect habituation (Fig. 6A). However, short-term olfactory habituation was impaired in *dysb*^{1} animals in trans with the dysbindin deficiency (*BSC 416*) (Fig. 6A) 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, Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013, Twick, Lee et al. 2014). We determined the requirement for Dysbindin within these neuronal subtypes by conditionally knocking-down dysbindin in a cell-type specific manner. We used *dysb* RNAi selectively expressed in the lateral interneurons and antennal lobe projection neurons with the LN1 and GH146-Gal4 drivers, respectively (Das, Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013). Dysbindin requirement in both cell types for short-term olfactory habituation, demonstrate that Dysbindin is necessary for olfactory memory formation (Fig. 6B). Moreover, the Dysbindin cell specific requirement is temporally limited to the adult olfactory circuit as determined by experiments that restricted Dysbindin knock-down to the adult circuit using the transcriptional repressor tub-Gal80^{ts}

(Fig. 6C, 18°C). Repression of Gal80^{ts} at the non-permissive temperature led to the expression of dysbindin-RNAi and impaired olfactory habituation phenotype (Fig. 6C, 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 projections neurons during adult *Drosophila* olfactory habituation.

We next asked if 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. 6D). 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. 6E). 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 is required irrespective of the synapse organization level analyzed or the central or peripheral character of a synapse. However, these 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 co-segregation of traits with genotypes. We analyzed genotype-to-phenotype pairs using single linkage hierarchical clustering (Fig. 7). We assigned a value of 0 to wild type and 1 to mutant phenotypes, respectively. Clustering revealed that a homozygous *Blos1* null genotype (Fig. 7A*Blos1-/-*) is phenotypically closer to a *Blos1* heterozygote (Fig. 7A*Blos1-/+*) than to homozygous *dysb* loss-of-function genotype (Fig. 7A*dysb-/-*). In contrast, the phenotypes observed in homozygous *dysb* loss-of-function null flies (Fig. 7A*dysb-/-*) co-segregated better with a *dysb, Blos1* transheterozygotes (Fig. 7A*Blos1/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. 7B). This analysis shows that an array of six genotypes and five traits identify as a minimum two non-overlapping phenotypic clusters within a collection of BLOC-1 loss-of-function mutations. Our findings demonstrate that alleles reducing the function of a 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.

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, Daniels et al. 2010, Guruharsha, Rual 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 (Fig. 2-3). 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. 6). We draw two conclusions from these findings that we would like to focus on. First, gene dosage reductions in two or more genetic loci affecting BLOC-1 do not phenocopy each other, nor do they confer a simple additive loss of function as has been described for recessive alleles. Rather, mutations to BLOC-1 are governed by

the dosage dependent hypothesis, consistent with reports of the complex genetic interactions well described in regulatory gene complexes (Birchler 2000, 2001) but not yet observed in relation to either membrane-trafficking complexes or neuropsychiatric disorders. Second, genotype-to-phenotype correlations observed in a trait following a gene pair analysis better, although not precisely, predict 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, Ophoff et al. 2009, Bassett, Scherer et al. 2010, Malhotra and Sebat 2012, Rapoport, Giedd et al. 2012, Moreno-De-Luca, Myers et al. 2013, Ahn, Gotay et al. 2014, Stefansson, Meyer-Lindenberg 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, Bottani 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, Kuo et al. 1999, Zhang, Li et al. 2002, Ciciotte, Gwynn et al. 2003, Li, Zhang et al. 2003, Gwynn, Martina et al. 2004, Starcevic and Dell'Angelica 2004, Yang, He et al. 2012). We postulate that olfactory habituation, a BLOC-1 recessive

phenotype, is at one end of this spectrum and requires fully assembled octamericBLOC-1, which would be disrupted by any BLOC-1 subunit mutation. Further, olfactory habituation mechanisms would be unaffected by BLOC-1 remnants (Fig. 8). 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 non-mutually 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. 8). Thus, some phenotypes may emerge from these BLOC-1 complex remnants leftover after uneven protein down-regulation 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 remnants, 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, Middleton et al. 2000, Purcell, Wray et al. 2009, Faludi and Mirnics 2011, Purcell, Moran et al. 2014). Here, we focus on the product of a gene associated to schizophrenia susceptibility and a modulator of cognition in normal humans, dysbindin. 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, Burdick 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. 6). 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,

Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013, Twick, Lee 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, as well as in both the prefrontal cortex and hippocampus of schizophrenia patients (Benes and Berretta 2001, Beasley, Zhang et al. 2002, Hashimoto, Volk et al. 2003, Hashimoto, Arion et al. 2008, Nakazawa, Zsiros et al. 2012). Second, impaired sensory habituation is a common manifestation in schizophrenia subjects (Geyer and Braff 1987, Braff, Grillon et al. 1992, Holt, Weiss et al. 2005, Williams, Blackford et al. 2013, Hu, He et al. 2014). In humans, sensory habituation defects are considered an intermediate, or 'endo-', phenotype. 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 philanthotoxin-induced synaptic homeostasis (Fig. 7). 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.

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. *dysb*[1], UAS-Dysb, UAS-Venus-dysbindin, $dysb^{rv}$, $dysb^{Df}$ were obtained from Graeme Davis (UCSF); $blos1^{ex2}$, $blos1^{ex65}$, and UAS-blos1 were obtained from Esteban Dell'Angelica (UCLA)(Cheli, Daniels et al. 2010). w^{1118}, Canton S, Elav-GAL4^{C155} 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. $Blos1^{EY}$ was obtained from Bloomington *Drosophila* Stock Center and UAS-dysb RNAi was from NIG.

For all physiological intracellular recordings, data was 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 -60mV and -90mV and the muscle input resistance was >5MΩ. For mEJP analysis and philanthotoxin experiments, intracellular recordings were performed in modified HL3 saline (in mM: NaCl 70, KCl 5, $CaCl_2$ 0.3, $MgCl_2$ 1.0, $NaHCO_3$ 10, Sucrose 115, Trehalose 5, BES [2 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid pH 7.2] 5). Severed motor neurons were taken up into a stimulating electrode and stimulated at 1Hz for 50s. For acute pharmacological homeostatic challenge, experiments were conducted as previously described (Dickman and Davis 2009, Dickman, Tong et al. 2012). Briefly, semi-intact preparations were maintained with the CNS, fat, and gut intact and perfused with Phillanthatoxin-433 (PhTx;

Sigma). A stock solution of PhTx was prepared (4mM in DMSO) and diluted for use to 4µM in modified HL3. Preparations were incubated for 10 minutes in PhTx, rinsed in modified HL3, and dissections were then completed. Recordings were only used if the mEJP amplitude following toxin incubation was ≤60% of baseline mEJP amplitude, indicative of the toxin gaining access to the muscle.

For vesicle pool separation experiments, physiological recordings were carried out in normal HL3 (in mM: NaCl 70, KCl 5, $CaCl_2$ 1.0, $MgCl_2$ 2.0, $NaHCO_3$ 10, Sucrose 115, Trehalose 5, BES [2 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid pH 7.2] 5). Prior to stimulation, animals were dissected in Ca^{2+}-free HL3 and incubated in 1µM bafilomycin A1 for 15 minutes. After incubation, severed motor neurons were taken up into a stimulating electrode and stimulated for 30 minutes at either 3Hz (low-frequency) or 10Hz (high-frequency) in the presence of 1µM bafilomycin A1 (Sigma Aldrich, Cat No. B1793). Bafilomycin A1 was prepared as a 1mM stock solution in DMSO and diluted for us at 1µM in normal HL3. Semi-intact preparations were prepared as described (Kim, Kumar et al. 2009).

For all electrophysiological experiments, a magnetic glass microelectrode horizontal puller (PN-30 Narishige) was used to prepare microelectrodes (30-70 MΩ resistance, backfilled with 3M KCl). Amplification of signals was achieved using Axoclamp900A. Signals were digitized using Digidata 1440A and recorded using Clampfit 10.1. Analysis was done in MiniAnalysis (Synaptosoft Inc.) and Microsoft Excel. For baseline evoked responses and homeostasis assays, the average EJP was divided by the average mEJP to determine quantal content. For

low- and high-frequency stimulation protocols, quantal content for each stimulated response was calculated, and then normalized as a percent of the first recorded response. Tau was calculated for the stimulation to 50% decay of initial response. Nonlinear summation correction was applied across all quantal content calculations.

Immunohistochemistry and confocal microscopy

Larval dissections, immunohistochemistry, and confocal microscopy were conducted according as previously described (Franciscovich, Mortimer et al. 2008). Wandering third instar female larvae were dissected in normal HL3, fixed in 4% paraformaldehyde for 1 hour, and stained with HRP –FITC for 2 hours at room temperature (1:500). An inverted 510 Zeiss LSM microscope was used for confocal imaging. At least 15 animals were counted per genotype.

Immunoprecipitation and mass spectrometry

To determine interactions between *Drosophila* BLOC-1 subunits, fly heads were prepared as previously described (Roos and Kelly 1998). Briefly, for each genotype, approximately 100 animals were flash frozen in liquid nitrogen, 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 $MgCl_2$, pH 7.4)+ 0.5% TritonX-100) with Complete anti-protease (catalog#11245200, Roche), 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 (BioRad, Villerica, CA). Proteins were resolved by SDS-PAGE on a 4-20% gel (Invitrogen) and immunoblot analysis was performed as previously described(Gokhale, Larimore et al. 2012, Ryder, Vistein et al. 2013). Dyna magnetic beads (catalog #110.31, Invitrogen) coated with antibody (catalog #, Invitrogen) and incubated for 2 h at 4°C with 500µg of protein extract. The beads were then washed 4–6 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 mass spectrometry protein identification by MS Bioworks as described(Gokhale, Larimore et al. 2012, Ryder, Vistein et al. 2013).

Short-term olfactory habituation

Short-term olfactory habituation was tested using a Y-maze apparatus as previously described (Das, Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013). Briefly, four-day-old adult flies were starved overnight and the naïve response to ethyl butyrate (EB, 10^{-3} 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 mL Eppendorf with 5% EB in paraffin oil with the lid perforated for 30 minutes. Animals were then tested for response following the 30 minutes period (post-test response). For conditional dysbindin knock-down experiments parental flies were reared at 18°C until eclosion. Newly eclosed flies (0-12 hrs) were shifted to 29°C, whereas the control flies were maintained at 18°C. After 4 days, flies were subjected to short-term habituation protocol, as described above.

Statistical and cluster analysis

Experimental conditions were compared using Synergy Kaleida-Graph, version 4.1.3 (Reading, PA), or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft, Vancouver, Canada). Tests are indicated in each figure. Cluster analysis was performed with Cluster 3.0 (http://rana.lbl.gov/EisenSoftware.htm)(Eisen, Spellman et al. 1998) and visualized using TreeView-1.1.6r4. Random genotype-phenotype pairs were generated with the engine random.org.

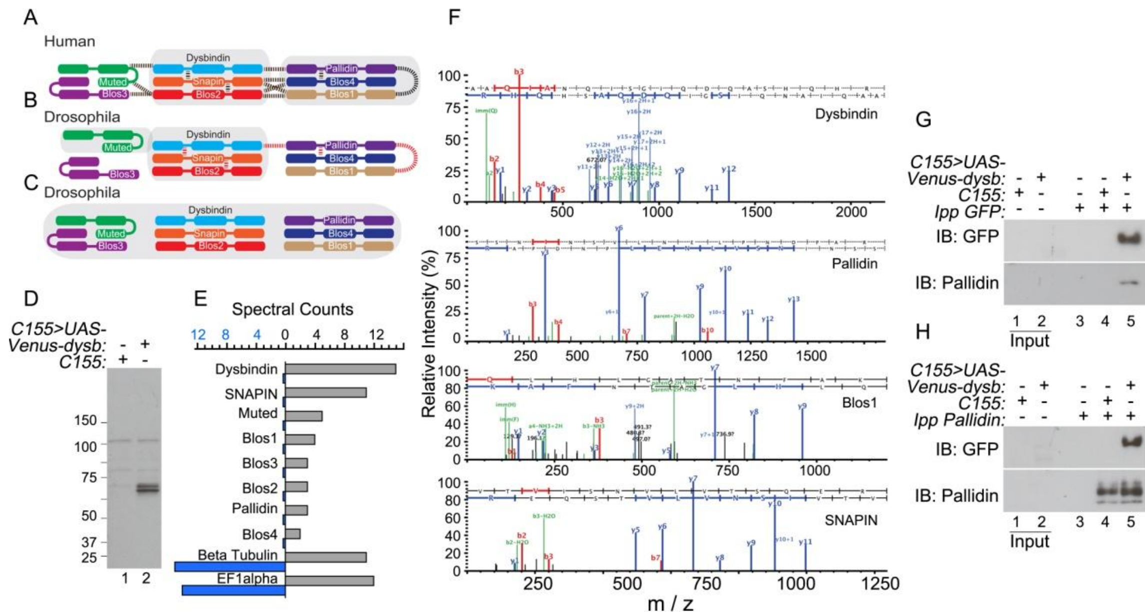

Figure 1. BLOC-1 assembles into an octameric complex in *Drosophila* neurons. Previously identified mammalian (A) and *Drosophila* (B) BLOC-1 subunit interactions. Dotted lines represent interactions identified by yeast two-hybrid (dotted lines), while shaded regions depict complex or subcomplexes formation based on immunoprecipitation or cosedimentation studies. Identification of *Drosophila* BLOC-1 subunits immunoprecipitating Venus-Dysbindin is consistent with octameric mammalian BLOC-1 architecture (C). Immunoblot with GFP antibodies confirms expression of the Venus-Dysbindin transgene from fly head lysates in animals expressing the transgene (lane 2) but not control animals (lane 1) (D). Lysates as shown in D were immunoprecipitated using GFP antibodies. Spectral counts of all eight BLOC-1 subunit orthologues were selectively enriched following immunoprecipitation with GFP antibodies from animals expressing the Venus-Dysbindin transgene (E, grey bars) compared to controls (E, blue bars). Proteins non-specifically bound to the GFP beads such as beta-tubulin and elongation factor 1alpha (EF1alpha) were represented with similar spectral counts in both samples (E). MS/MS peptide sequencing of select immunoprecipitated *Drosophila* BLOC-1 subunit orthologues (F). Specific detection of BLOC-1 ortholog Pallidin by immunoblot in Venus-Dysbindin-expressing fly head lysates immunoprecipitated with GFP antibodies (G, lane 5). Pallidin antibodies precipitate Venus-Dysbindin from Venus-Dysbindin expressing fly head lysates detected with GFP antibodies (H, lane 5).

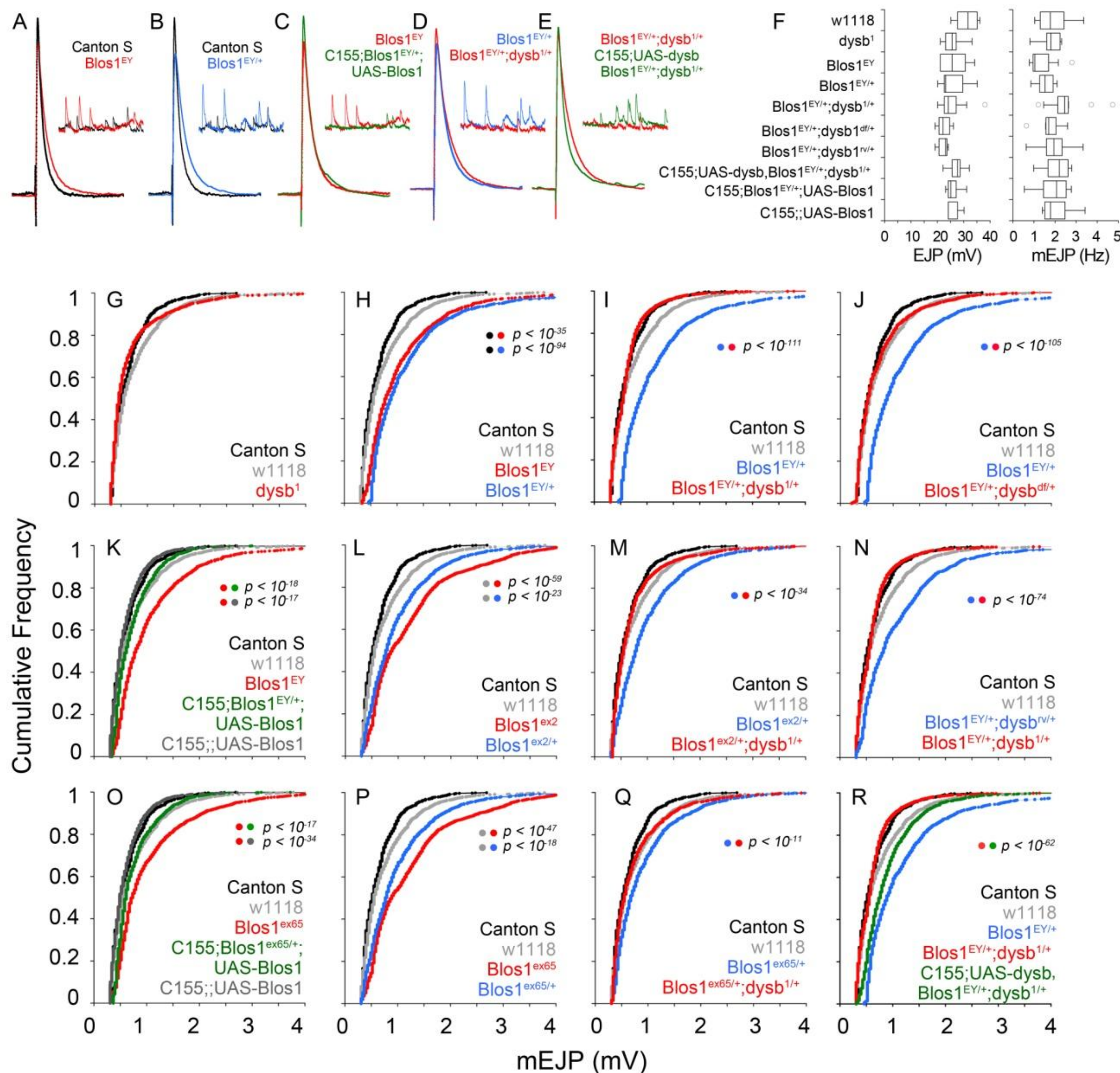

Figure 2. BLOC-1 presynaptically regulates quantal content at the *Drosophila* NMJ. A-E) Representative EJP and mEJP traces showing Blos1 null (A, red, Blos1^{EY}) and Blos1 heterozygote (B, blue, Blos1$^{EY/+}$) increase mEJP but not EJP amplitude compared to control (Canton S, black). Presynaptic expression of UAS-Blos1 in a single copy *Blos1* loss (C, green) or single copy loss of *Blos1* in trans with the *dysb*^{1} mutation (D, red) rescues mEJP amplitude as compared to *Blos1* heterozygote (C-D, blue). Presynaptic expression of dysb in *Blos1*^{EY};*dysb*^{1} transheterozygote (E, green) increases mEJP amplitude compared to the transheterozygote (E, red). No changes in EJP amplitude or mEJP frequency were observed in any of the genotypes (F). G-R) Event size plotted against frequency of event for BLOC-1 loss of function allelic combinations. Shift in the curve to the right indicates larger events in that genotype. All comparisons in F were performed with One-Way ANOVA followed by Bonferroni multiple comparison test. G to R comparisons were performed by Kolmogorov–Smirnov test. F to R data were obtained from 7-11 animals per genotype. G to R plots graph between 992 and 2880 randomly selected mEJP events.

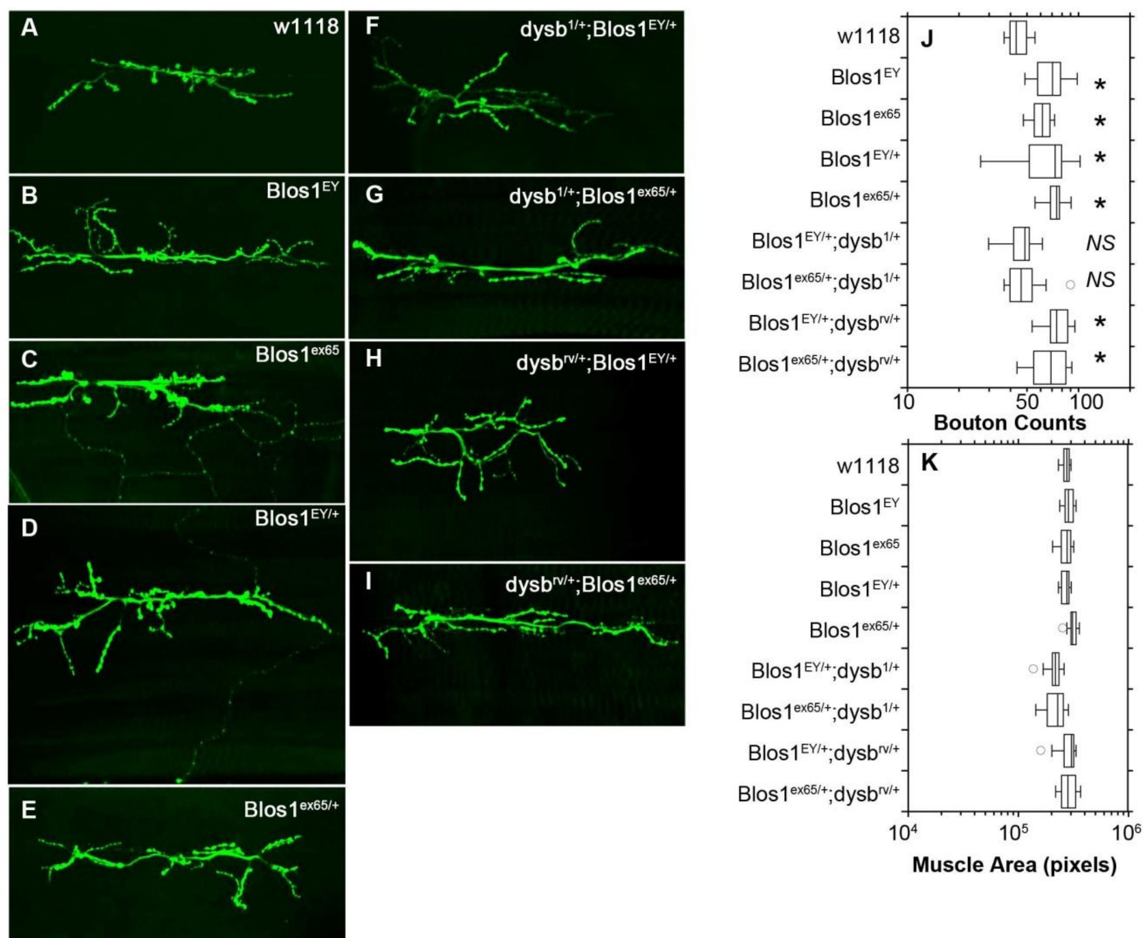

Figure 3. Normal synaptic morphology requires BLOC-1 function. A-I) Representative images of HRP immunofluorescence at the 6/7 synapse in *Drosophila* for indicated genotypes. J) Significant increases in the number of synaptic boutons in BLOS1 null animals (Blos1^{EY}, Blos1^{ex65}) and animals carrying single copy loss of BLOS1 (Blos1$^{EY/+}$, Blos1$^{ex65/+}$, Blos1$^{EY/rv}$, Blos1$^{ex65/rv}$), but no significant changes in synaptic bouton numbers in Blos1$^{EY/+}$;dysb$^{1/+}$ or Blos1$^{ex65/+}$;dysb$^{1/+}$ transheterozygotes. K) Muscle area across all genotypes was unaffected. All comparisons were performed with One-Way ANOVA followed by Dunnett's multiple comparison test. Data were obtained from 10-14 animals per genotype. * p between 0.0019 and 0.045 and ns indicate non-significant *p* values.

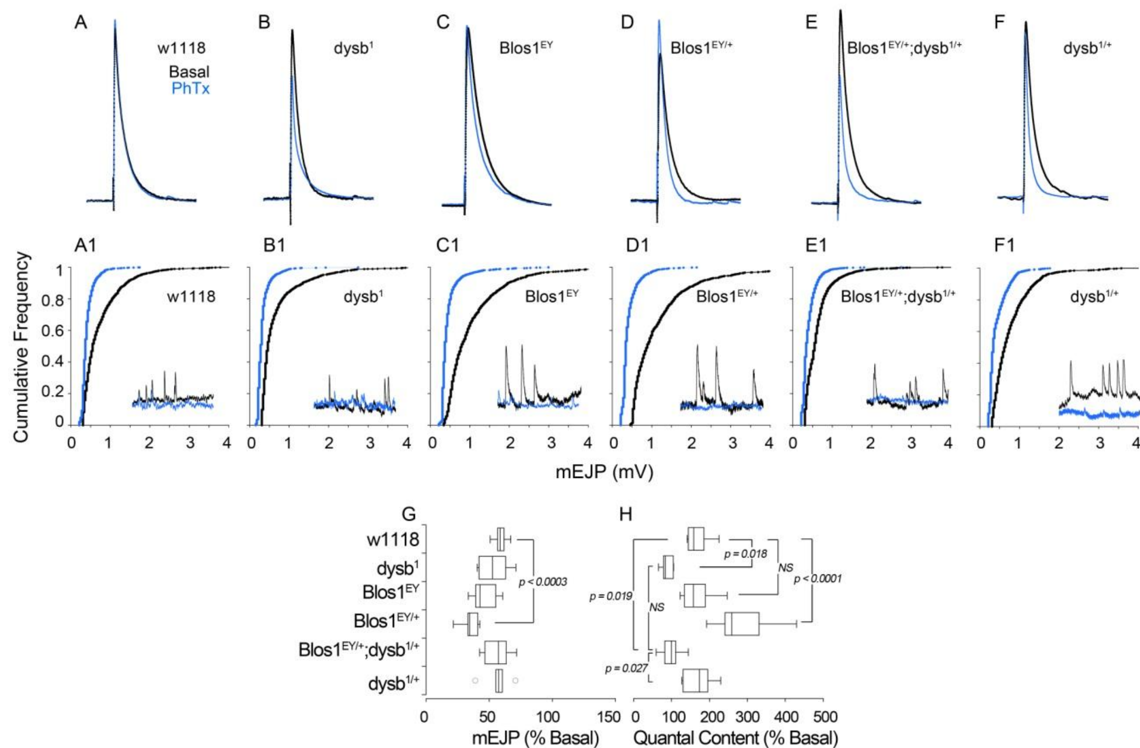

Figure 4. BLOC-1 gene-dosage regulates synaptic homeostasis. A-F) Representative EJP traces. Black indicates baseline stimulated response; blue indicates response following acute 10-minute incubation with 4uM PhTx for each genotype. A1-F1) Reduced mEJP amplitudes following PhTx incubation (blue) compared to baseline (black). Representative mEJP traces shown in inset. G) Reduction in mEJP amplitude following toxin incubation. H) w1118 control, Blos1^{EY}, and Blos1$^{EY/+}$ animals display robust homeostatic increase in quantal content following toxin incubation, while dysb^{1} and Blos1$^{EY/+}$;dysb$^{1/+}$ animals did not. A1 to F1 plots graph between 909 and 2888 randomly selected mEJP events. All comparisons in G and H were performed with One-Way ANOVA followed by Bonferroni's multiple comparison. Data were obtained from 6-11 animals per genotype.

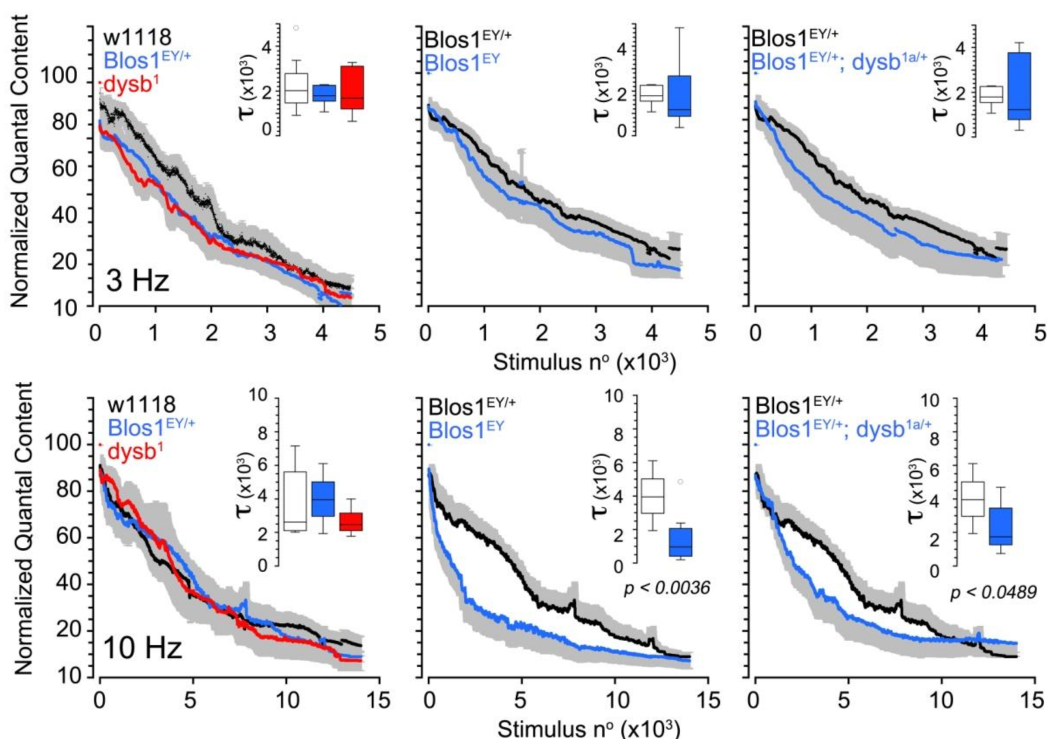

Figure 5. BLOC-1 gene-dosage regulates synaptic vesicle pool properties. Animals were stimulated at low frequency (3 Hz, A-C) and high frequency (10 Hz, D-F) in the presence of 1uM bafilomycin A1. Inset shows stimulation to 50% depletion compared to response at stimulation 0. No changes in rate of vesicle pool depletion were observed across genotypes at low frequency (A-C). At high frequency, no changes were observed in vesicle depletion rate in $dysb^1$ (D, red), or $Blos1^{EY/+}$ (D, blue) compared to control (D, w1118, black). $Blos1^{EY}$ (E, blue) and $Blos1^{EY/+}$;$dysb^{1/+}$ transheterozygote (F, blue) both displayed significantly faster vesicle depletion compared to the $Blos1^{EY/+}$ (E-F, black). All comparisons in F and G were performed with One-Way ANOVA followed by Fishers's multiple comparison. Data were obtained from 6-9 animals per genotype.

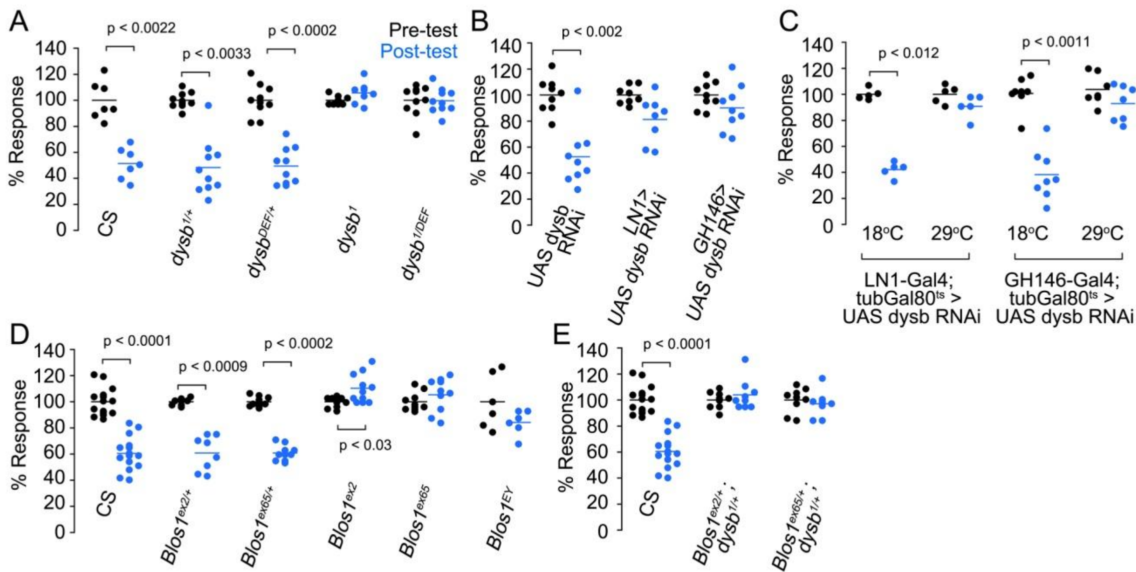

Figure 6. BLOC-1 is required at local interneurons and projection neurons for short-term olfactory habituation in *Drosophila*. Following aversive odor exposure, control (Canton S, CS) or single copy loss of function ($dysb^{1/+}$, $dysb^{DF/+}$) animals show significant avoidance behavior, represented as % Response, compared to 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 local interneurons (LN1 driver) or projection neurons (GH146 driver) is sufficient to prevent normal post-exposure avoidance response. C) Inhibition of dysbindin RNAi expression in the local interneurons or the projection neurons using the temperature-sensitive repressor $tubGal80^{ts}$ at the permissive temperature (18°C) allowed for normal olfactory habituation. Repression of $tubGal80^{ts}$ at the non-permissive temperature (29°C) led to impaired odorant avoidance response. D) Blos1 homozygous mutations but not single copy loss prevent learned odorant avoidance behavior. (E) Blos1 mutations expressed in trans with $dysb^{1}$ prevent learned odorant avoidance response. Dot plots represent each animal per genotype. Comparisons were made with Wilcoxon-Mann-Whitney test.

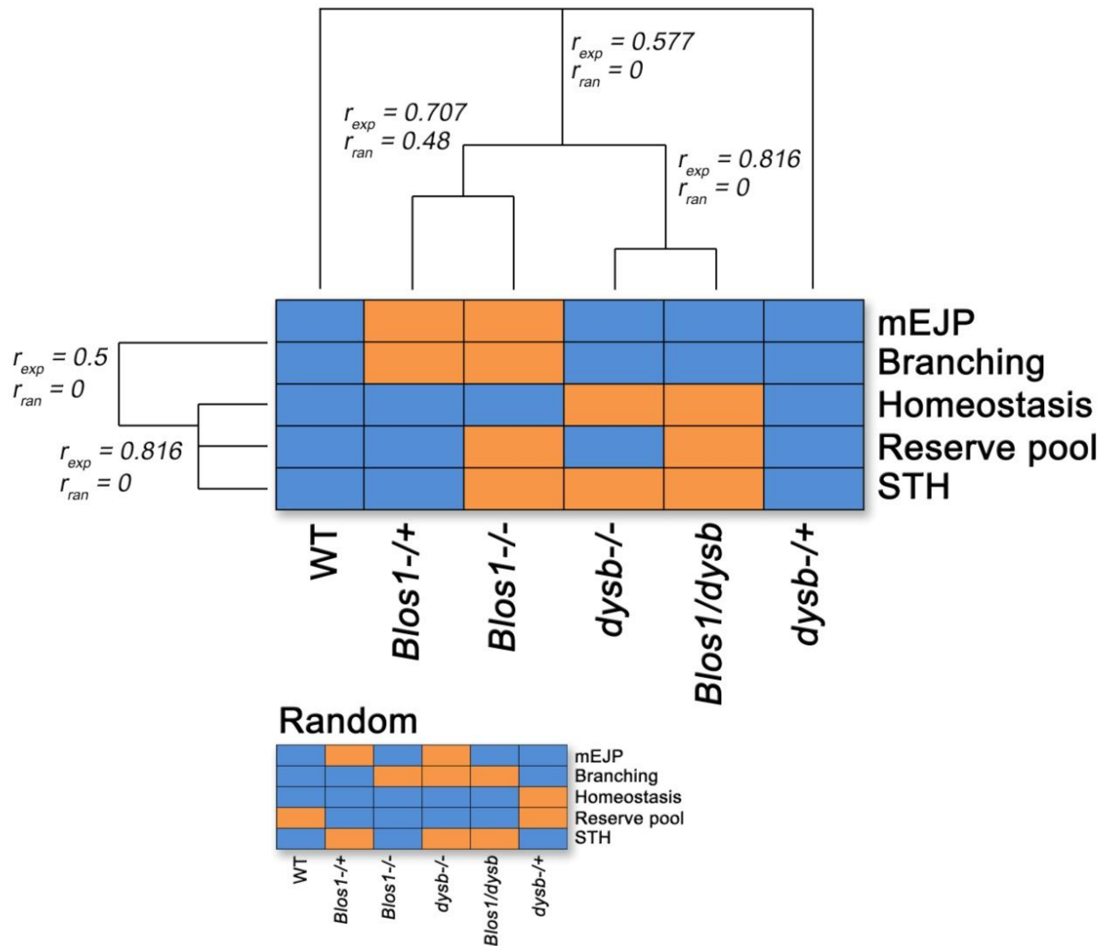

Figure 7. Hierarchical Clustering Analysis of BLOC-1 Genotype and their Associated Phenotypes. Genotype-to-phenotype pairs were analyzed by single linkage hierarchical clustering. Phenotypes are assigned a value of 0 to wild type (blue) and 1 to mutant phenotypes (orange), respectively. A depicts experimentally generated genotype-to-phenotype pairs. B represents randomly assigned genotype-to-phenotype pairs. Hierarchical clustering analysis was performed for A-B and clusters 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.

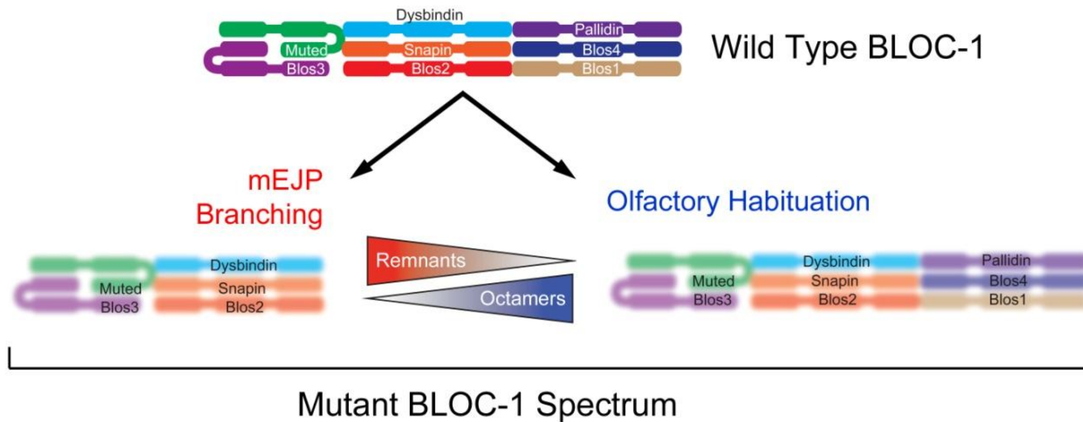

Figure 8. How do BLOC-1 mutations produce divergent synaptic phenotypes? Model depicts the wild type BLOC-1 complex and two outcomes of gene loss-of-function alleles on the levels and putative architecture of BLOC-1 and remnants (blurred structures). Mutations induce a down-regulation of the whole BLOC-1 octamer (bottom left) and/or generate low levels of BLOC-1 remnants (bottom right). The extent of these two molecular outcomes would dependent of the mutations affecting BLOC-1 subunit genes. Phenotypes emerge either because of down-regulation of the BLOC-1 octamer or the appearance of remnants. See discussion for details.

Supplementary Table 1				
Fig. no.	**Genotype**	**Negative RI values for STH experiments**		
		Naïve (N)	**Exposed (N)**	***p*-value**
6A	**CS**	0.63 ± 0.03 (7)	0.33 ± 0.03 (7)	0.002141
	dysb $^{1/+}$	0.60 ± 0.01 (10)	0.29 ± 0.04 (10)	0.0003214
	dysb $^{DEF/+}$	0.58 ± 0.02 (10)	0.29 ± 0.03 (10)	0.0001796
	dysb ^{1}	0.64 ± 0.008 (8)	0.68 ± 0.02 (8)	NS
	dysb $^{1/DEF}$	0.79 ± 0.03 (10)	0.79 ± 0.02 (10)	NS
6B	**CS > UAS-*dysb* RNAi**	0.62 ± 0.03 (9)	0.33 ± 0.05 (9)	0.001976
	***LN1*-GAL4 > UAS-*dysb* RNAi**	0.64 ± 0.02 (8)	0.52 ± 0.04 (8)	NS
	***GH146*-GAL4 > UAS-*dysb* RNAi**	0.69 ± 0.02 (9)	0.62 ± 0.04 (9)	NS
6C	***LN1*-GAL4; *tub*-GAL80** ^{ts} **> UAS-*dysb* RNAi - 18˚C**	0.64 ± 0.009 (5)	0.27 ± 0.01 (5)	0.01193
	***LN1*-GAL4; *tub*-GAL80** ^{ts} **> UAS-*dysb* RNAi - 29˚C**	0.69 ± 0.02 (5)	0.63 ± 0.02 (5)	NS
	***GH146*-GAL4, *tub*-GAL80** ^{ts} **> UAS-*dysb* RNAi - 18˚C**	0.64 ± 0.02 (9)	0.25 ± 0.04 (9)	0.001084
	***GH146*-GAL4, *tub*-GAL80** ^{ts} **> UAS-*dysb* RNAi - 29˚C**	0.68 ± 0.04 (8)	0.61 ± 0.03 (8)	NS
6D	**CS**	0.64 ± 0.02 (15)	0.39 ± 0.02 (15)	< .0001
	blos1 $^{ex2/+}$	0.63 ± 0.006 (8)	0.38 ± 0.03 (8)	0.000891
	blos1 $^{ex65/+}$	0.62 ± 0.007 (10)	0.38 ± 0.01 (10)	0.0001717
	blos1 ^{ex2}	0.60 ± 0.007 (11)	0.66 ± 0.02 (11)	0.02771
	blos1 ^{ex65}	0.57 ± 0.01 (11)	0.60 ± 0.02 (11)	NS
	blos1 ^{EY}	0.56 ± 0.05 (6)	0.47 ± 0.02 (6)	NS
6E	**CS**	0.64 ± 0.02 (15)	0.39 ± 0.02 (15)	< .0001
	blos1 $^{ex2/+}$ ***; dysb*** $^{1/+}$	0.63 ± 0.01 (9)	0.66 ± 0.02 (9)	NS
	blos1 $^{ex65/+}$ ***; dysb*** $^{1/+}$	0.62 ± 0.02 (9)	0.60 ± 0.02 (9)	NS

 # RI values are ± SEM

Response index (RI) was calculated for naive- and odor-exposed flies, as the fraction of flies in the odorant compartment minus the fraction of flies in the air compartment divided by the total number of files that participated in the experiment.

See J Neurosci. 2013 Oct 16;33(42):16576-85. for details

Supplementary Table 1. Summary of results for olfactory short-term habituation experimental data shown in Figure 6.

CHAPTER III

NSF Acts Downstream of the Schizophrenia Susceptibility Factor, Dysbindin, to Regulate Synaptic Homeostasis.

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Running Title: NSF is Downstream of Dysbindin

*These authors contributed equally to this work.

Under Review, *PNAS*

ABSTRACT

Synaptic homeostatic mechanisms maintain cellular and circuit excitability set points. Dysbindin, a schizophrenia susceptibility factor and subunit of the BLOC-1 complex, is required presynaptically for synaptic homeostatic plasticity at the *Drosophila* neuromuscular junction, suggesting disruption of synaptic homeostasis as a potential mechanism for neurodevelopmental disorders. However, molecular pathways mediating synaptic homeostasis downstream of dysbindin-BLOC-1 remain mostly unknown. Here, we identified the N-Ethylmaleimide Sensitive Factor (NSF) as a factor sensitive to BLOC-1 deficiency. BLOC-1 interacts independently with NSF. To test the hypothesis that NSF is necessary for dysbindin function, we examined a role for NSF in dysbindin-BLOC-1-dependent synaptic homeostatic compensation at the *Drosophila* neuromuscular junction. While mutations in *dysbindin* preclude philanthotoxin-induced homeostatic plasticity, a phenotype rescued by presynaptic expression of *dysbindin*, neuron-specific expression of *dNSF1* fully rescued this defect. Our results demonstrate that NSF resides downstream of dysbindin-BLOC-1, and that presynaptic homeostatic mechanisms require synaptic vesicle fusion machinery.

SIGNIFICANCE STATEMENT

One of the schizophrenia pathogenic hypotheses postulates defects in the communication between the pre- and post-synapse. Here we identified that a synaptic communication impairment caused by a mutation in a schizophrenia susceptibility gene is reversed by selective expression of membrane fusion machinery components at the pre-synapse. These findings identify a two-gene synaptic schizophrenia susceptibility pathway regulating neurotransmitter secretion.

INTRODUCTION

Neural circuits and their constituent neurons are capable of regulating their synaptic activity in the face of changing demands. At individual synapses, pre- and post-synaptic mechanisms combine in a process known as synaptic homeostasis to maintain synaptic activity within a set range in order to elicit the appropriate synaptic communication and postsynaptic response (Turrigiano 2008, Davis 2013). Genes that lead to defects in synaptic homeostasis are also associated with or cause a subset of neurodevelopmental disorders (Wondolowski and Dickman 2013). Thus, abnormal synaptic homeostasis could be a central link in the chain of events leading from genetic mutations to the phenotypes observed in complex neurodevelopmental disorders (Wondolowski and Dickman 2013). This concept is illustrated by the schizophrenia susceptibility factor dysbindin. Loss-of-function mutations in *Drosophila dysbindin* preclude a form of presynaptic homeostasis elicited by blockage of postsynaptic receptors at the neuromuscular junction (Dickman and Davis 2009, Dickman, Tong et al. 2012). In addition, Snapin, a polypeptide that associates with dysbindin and SNAREs (Ilardi, Mochida et al. 1999, Starcevic and Dell'Angelica 2004, Tian, Wu et al. 2005), is also required for presynaptic homeostatic plasticity in the fly (Dickman, Tong et al. 2012). This suggests that the network of protein-protein interactions engaged by dysbindin is necessary for presynaptic plasticity mechanisms. Dysbindin associates with seven other polypeptides, one of them Snapin, to form an octameric complex known as the biogenesis of lysosome-related organelles complex 1 (BLOC-1) (Starcevic and Dell'Angelica 2004, Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011). Moreover, the BLOC-1 complex also associates

with monomeric SNARES, regulating their subcellular distribution and content in the hippocampus (Salazar, Craige et al. 2006, Ghiani, Starcevic et al. 2009, Newell-Litwa, Chintala et al. 2010). This evidence suggests that dysbindin-BLOC-1-dependent synaptic homeostasis mechanisms might require components of the SNARE-mediated fusion apparatus for normal plasticity.

The human dysbindin polypeptide and its gene, *DTNBP1*, are of particular interest as polymorphisms in *DTNBP1* associate with an increased risk of schizophrenia, a neurodevelopmental disorder. *DTNBP1* genetic polymorphisms are risk factors for schizophrenia onset particularly in the childhood form of this disorder, a more severe version of schizophrenia than the adult affliction (Straub, Jiang et al. 2002, Van Den Bogaert, Schumacher et al. 2003, Luciano, Miyajima et al. 2009, Markov, Krug et al. 2009, Markov, Krug et al. 2010, Mechelli, Viding et al. 2010, Cerasa, Quattrone et al. 2011, Fatjó-Vilas, Papiol et al. 2011, Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011, Tognin, Viding et al. 2011, Wolf, Jackson et al. 2011, Ayalew, Le-Niculescu et al. 2012). Post-mortem studies of adult schizophrenia brains reveal a reduction in dysbindin transcripts and protein (Talbot, Eidem et al. 2004, Weickert, Rothmond et al. 2008). In fact, nearly 80% of affected subjects possess reduced dysbindin protein content in neuroanatomical regions affected by this disorder (Talbot, Eidem et al. 2004). These genetic and neuropathological findings in humans strongly argue for dysbindin and/or dysbindin interactors as penetrant, loss-of-function links in the chain of events associated to schizophrenia development.

We screened for proteomic modifications triggered by dysbindin-BLOC-1 loss-of-function in mammalian neuronal cells by unbiased quantitative mass spectrometry with stable isotope labeling with aminoacids in cell culture (SILAC). Our screen prominently identified components of the fusion machinery, including select SNAREs and NSF, as factors whose content is sensitive to BLOC-1 loss-of-function in diverse cell types, including neurons. We tested the hypothesis that that factors sensitive to BLOC-1 perturbation would converge on to a dysbindin-BLOC-1-mediated mechanism. We focused on NSF, the most downstream component of the vesicle membrane fusion machinery, and asked whether NSF participates in dysbindin-BLOC-1-dependent synaptic homeostatic compensation at the *Drosophila* neuromuscular junction. As previously reported, we observed that mutations in fly *dysbindin* precluded the establishment of homeostatic synaptic plasticity induced by blockage of postsynaptic receptors at the neuromuscular junction, a phenotype that we readily rescued by presynaptic expression of Dysbindin (Dickman and Davis 2009, Dickman, Tong et al. 2012). However, as predicted from our hypothesis, neuron-specific expression of *dNSF1*, the gene encoding *Drosophila* NSF, rescued this plasticity defect to the same extent as Dysbindin re-expression in the presynaptic compartment. Our results demonstrate that NSF resides downstream of dysbindin-BLOC-1, and that presynaptic homeostatic mechanisms require synaptic vesicle fusion machinery. We propose that defects in synaptic homeostasis due to reduced levels of dysbindin and dysbindin interactors, such as NSF, contribute to the synaptic pathology that characterizes schizophrenia.

RESULTS

To further understand the role played by dysbindin-BLOC-1 in synaptic homeostasis, we first profiled the cellular proteome from control and shRNA BLOC-1 down-regulated neuroblastoma cells. Quantitative mass spectrometry using stable isotope labeling by aminoacids in cell culture (SILAC) identified proteins sensitive to BLOC-1 down-regulation. These included subunits of the BLOC-1 complex (Bloc1s5 muted and Bloc1s4 cappuccino) and vesicle fusion machinery components (munc18, tomosyn, syntaxin 7 and 17, SNAP25, VAMP7, and NSF, Fig.1A and SFig.1)(Rizo and Südhof 2012). BLOC-1 complex subunits along with the fusion machinery form a predicted network of protein-protein/genetic interactions (Fig. 1B) where NSF is the most downstream component of the fusion machinery we identified. Thus we focused on this factor (Rizo and Südhof 2012).

We first confirmed that NSF is a BLOC-1-sensitive factor. We found cellular levels of NSF reduced in BLOC-1 down-regulated SH-SY5Y neuroblastoma cells (Figs. 1C-D). Down-regulation of BLOC-1 was achieved through shRNA targeted against BLOC-1 subunits Bloc1s5 muted, Bloc1s6 pallidin, or Bloc1s8 dysbindin and confirmed by a reduction in Bloc1s8 dysbindin on immunoblots (Figs. 1C-D). BLOC-1 loss-of-function also reduced NSF cellular levels in human neurons differentiated from iPSC cells (SFig.2) and neuroectodermal cell lines (Figs. 1E-F). Moreover, reduced NSF content in neuroectodermal cells carrying BLOC-1 null alleles *Bloc1s5*$^{mu/mu}$ or *Bloc1s6*$^{pa/pa}$ (Setty, Tenza et al. 2008), is increased by re-expression of the missing subunit (Fig. 1E-F). NSF transcripts were unaffected

by BLOC-1 subunit mutations (not shown). Furthermore, decreased NSF expression in Bloc1s6 pallidin shRNA-treated cells is not due to reduced VAMP7 levels since exogenous expression of VAMP7 did not rescue the NSF phenotype (SFig.3). These results establish NSF as a BLOC-1 sensitive factor.

We next asked whether changes in NSF and SNARE levels detected in BLOC-1 deficiency reflected molecular associations of BLOC-1 with components of the fusion apparatus. To determine if BLOC-1, SNAREs and NSF might exist in complex together, we analyzed their sedimentation in sucrose gradients. The BLOC-1 complex, detected with Bloc1s8 dysbindin antibodies, mostly co-sediments with NSF, with minimal overlap with the SNAREs VAMP7, SNAP25, and SNAP29 (Fig. 2A-B and SFig4. A-C). Endogenous or recombinant forms of these three SNARES co-precipitate with the BLOC-1 complex despite the low co-sedimentation of SNAREs and BLOC-1 (SFig.4D(Ryder, Vistein et al. 2013)). Similarly, endogenous NSF associates with BLOC-1 complexes containing FLAG-tagged dysbindin (Fig. 2C). FLAG antibodies precipitated the BLOC-1 subunit Bloc1s6 pallidin together with NSF (Fig. 2C, lane 2). This coprecipitation was prevented by addition of an excess of FLAG peptide (Fig. 2C lane 3). Conversely, antibodies against NSF precipitated endogenous NSF together with Bloc1s6 pallidin (Fig. 2D lane 2). Furthermore, an excess of recombinant full length GST-NSF prevented NSF-BLOC-1 copurification (Fig. 2D lane 3).

We conducted an unbiased identification of fusion machinery components associated with NSF-BLOC-1 complexes isolated using anti-NSF antibodies by mass spectrometry (Fig. 2E). In the absence of GST-NSF (Fig. 2E white bars), we

found molecules known to interact with BLOC-1, such as subunits of the adaptor complex AP-3 and components of the HOPS complex (Salazar, Zlatic et al. 2009). α-SNAP and low levels of two SNARES, YKT6 and syntaxin 5, were the only component of the fusion apparatus co-precipitating with endogenous NSF. In contrast, the addition of GST-NSF increased the content NSF bound to beads 17-fold, which in turn recruited three SNAP isoforms from cell lysates (24-fold increase), and increased the number of SNAREs identified to eleven. Among these were the BLOC-1-sensitive SNAREs SNAP29 and syntaxin 7. The increase in these fusion machinery components bound by GST-NSF occurred even though there was no increase in BLOC-1 binding observed (Fig. 2E). These results and the co-sedimentation profile of SNAREs, NSF, and BLOC-1 indicate that BLOC-1 forms complexes preferentially with either NSF or SNAREs.

The effect of the BLOC-1-NSF interaction on vesicle fusion was next tested in *Drosophila* S2 and HeLa cells. We asked whether BLOC-1 loss-of-function would impair NSF dependent fusion mechanisms. We assessed the effect of BLOC-1 down-regulation on the constitutive secretion of a pulse of GFP released from the endoplasmic reticulum of *Drosophila* S2 and HeLa cells (Gordon, Bond et al. 2010) (SFig. 5). Depletion of *Drosophila* syntaxin 5 using RNAi or treatment with brefeldin A prevented secretion of a pulse of GFP(Gordon, Bond et al. 2010), yet down-regulation of either *Drosophila* or human BLOC-1 subunits dysbindin, muted, or pallidin did not affect constitutive GFP secretion along the exocytic route. Thus, we excluded global effects of BLOC-1 deficiency on NSF-dependent processes.

We next asked whether NSF function is required for dysbindin-dependent phenotypes at the *Drosophila* neuromuscular junction. This synapse undergoes acute synaptic homeostatic compensation in response to the blockage of post-synaptic glutamatergic receptors with Philanthotoxin-433 (PhTx)(Davis 2013). Homozygotic mutations in BLOC-1 subunits *dysbindin* and *snapin* each block Philanthotoxin-induced homeostatic increase in quantal content, while heterozygotic mutations have no effect(Dickman and Davis 2009, Dickman, Tong et al. 2012). However, double heterozygotic mutations in *dysbindin* and *snapin* also reduce homeostatic compensation compared to wild type(Dickman and Davis 2009, Dickman, Tong et al. 2012). This suggested to us that this paradigm would be ideal for testing genetic interactions between dysbindin-BLOC-1 and NSF. Our results show that wild type synapses increase their quantal content by 182 ± 14.8 % following PhTx treatment (Fig. 3I). In contrast, and consistent with previous reports, animals carrying mutations in *dysbindin* (*dysb*^{1}) did not display homeostatic increase in quantal content (94.9 ± 13.5%, Fig. 3B-C and I). Neuron-specific expression of dysbindin rescued the *dysb*^{1} synaptic homeostasis defect (c155-GAL4;UAS-dysbindin;*dysb*^{1}, Figs. 3D,I). Strikingly, presynaptic expression of dNSF1-FLAG rescued the *dysb*^{1} synaptic homeostasis defect (c155-GAL4;UAS-dNSF1-FLAG, *dysb*^{1}) to the same extent as presynaptic dysbindin rescue (Fig. 3 compare D-E and G). Presynaptic addition of either dysbindin or dNSF1 restored homeostatic quantal content increase to wild type levels, 182.9 ± 17.5% and 171.3 ± 10.4%, respectively (Fig. 3I). These results demonstrate that dysbindin and NSF genetically interact in a molecular pathway necessary for synaptic homeostasis at the presynapse.

DISCUSSION

Here, we identified a protein-protein interaction network centered around a schizophrenia susceptibility gene, which encompasses molecules implicated in neurodevelopmental disorders, and tested if these network constituents are necessary for synaptic plasticity. These predictions emerge from the proposed concept that abnormal synaptic homeostasis could be a central phenotype in neurodevelopmental disorders, bridging single gene molecular defects with the observed cognitive and anatomical phenotypes (Wondolowski and Dickman 2013). We tested this hypothesis by focusing on the schizophrenia susceptibility gene *DTNBP1* and its product dysbindin.

We screened for proteins whose cellular content is sensitive to genetic reduction of dysbindin and its closely associated BLOC-1 subunits. Our proteomic search prominently highlights components of the vesicle fusion apparatus: munc18, tomosyn, NSF, and the SNAREs syntaxin 7, syntaxin 17, SNAP23, 25, 29, and VAMP7. Importantly, most of the aforementioned vesicle fusion machinery components have been implicated by genomic and post-mortem studies in several neurodevelopmental disorders, including schizophrenia (Thompson, Sower et al. 1998, Mirnics, Middleton et al. 2000, Saito, Guan et al. 2001, Halim, Weickert et al. 2003, Behan, Byrne et al. 2009, Gil-Pisa, Munarriz-Cuezva et al. 2012), intellectual disability (Hamdan, Piton et al. 2009), and autism spectrum disorder (Matsunami, Hadley et al. 2013, Cukier, Dueker et al. 2014). Our strategy is validated by the identification of proteins previously known to be down-regulated in null alleles of BLOC-1 subunits and/or known to interact with

BLOC-1. These proteins include subunits of the BLOC-1 complex (Bloc1s5 muted and Bloc1s4 cappuccino) and the SNARE VAMP7 (Li, Zhang et al. 2003, Starcevic and Dell'Angelica 2004, Salazar, Craige et al. 2006, Newell-Litwa, Chintala et al. 2010, Ghiani and Dell'angelica 2011, Mullin, Gokhale et al. 2011, Yang, He et al. 2012). We further authenticated these fusion machinery components as part of a dysbindin-BLOC-1 network by a) co-immunoprecipitation of a fusion machinery component with dysbindin-BLOC-1 subunits, and/or b) down-regulation of a fusion machinery component after genetic or shRNA mediated reduction of dysbindin-BLOC-1 subunits. We centered on NSF since it is the most downstream component of the vesicle fusion machinery. We found that NSF associates with dysbindin and BLOC-1 subunits, and that NSF cellular levels are decreased following shRNA mediated or genomic reduction of BLOC-1 complex members. This phenotype was observed consistently in neuroblastoma and embryonic kidney human cells, neuroectodermal cells, and iPSC-derived human neurons.

The functional consequences of NSF reduction in BLOC-1 loss-of-function become evident only when the synapse is challenged. Constitutive secretion in *Drosophila* or mammalian non-neuronal cells is unaffected, as are spontaneous and evoked neurotransmission at the *Drosophila* neuromuscular junction. However, a necessity for NSF in BLOC-1 loss-of-function phenotypes can be localized to a presynaptic homeostatic mechanism, which is engaged when postsynaptic receptors are blocked with philanthotoxin. After a brief incubation with philanthotoxin, the resultant reduction in post-synaptic signal transduction

rapidly induces a compensatory increase in quantal content, a response known as presynaptic homeostatic plasticity (Davis 2013, Frank 2014). This adaptive compensatory mechanism is precluded by *dysbindin* mutations, and can be rescued by presynaptic expression of Dysbindin (Dickman and Davis 2009). However, we were able to rescue this phenotype in the *dysbindin* mutants to the exact same extent through presynaptic expression NSF. These findings demonstrate that NSF function resides downstream of dysbindin-BLOC-1 and suggest that dysbindin-NSF-dependent mechanisms are required for a restricted pool of vesicle fusion events. Dysbindin and its interacting BLOC-1 subunits, as well as all of the fusion machinery identified in our studies with the exception of syntaxin 17, reside in synaptic vesicles (Salazar, Craige et al. 2006, Takamori, Holt et al. 2006, Newell-Litwa, Salazar et al. 2009, Newell-Litwa, Chintala et al. 2010). Thus a functionally and/or biochemically defined subpopulation of synaptic vesicles may mediate compensatory synaptic homeostatic responses (Muller, Liu et al. 2012, Ramirez, Khvotchev et al. 2012, Bal, Leitz et al. 2013, Morgan, Comstra et al. 2013).

How does the BLOC-1-NSF interaction affect synaptic plasticity mechanisms? We believe a model integrating our findings has to consider three key elements. First, BLOC-1 subunits reside at endosomes as well as on synaptic vesicles in presynaptic terminals in neurons (Di Pietro, Falcon-Perez et al. 2006, Setty, Tenza et al. 2007, Ryder and Faundez 2009, Mullin, Gokhale et al. 2011). Second, BLOC-1 binds monomeric SNAREs rather than tetrahelical SNARE bundles in vitro (Ghiani, Starcevic et al. 2009). Finally, NSF and SNAREs independently

bind to dysbindin-BLOC-1. Thus, we propose a scenario where BLOC-1 bound to a single SNARE, perhaps for SNARE sorting into vesicles, is resolved by NSF, making SNAREs permissive for vesicle fusion. Therefore, when NSF levels are reduced, such as in dysbindin or BLOC-1 mutants, SNARE-dependent mechanisms might be adversely affected (SFig.6). Resultant defects in synaptic plasticity mediated by the fusion apparatus downstream of dysbindin-BLOC-1 complexes ultimately contribute to the pathogenesis of neurodevelopmental disorders such as schizophrenia. Changes in the cellular proteome due to mutations in a schizophrenia susceptibility gene guided us to identify phenotypes connecting the pre- and post-synaptic cells. We propose this strategy is a simple yet rigorous approach to unravel mechanisms of complex neurodevelopmental disorders.

MATERIALS AND METHODS

Cell culture

SH-SY5Y (ATCC) cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 100 g/ml penicillin and streptomycin (Hyclone) at 37°C in 10% CO2. SH-SY5Y cell line carrying 3x-FLAG Dysbindin (catalog #EX-Mm12550-M12) was previously described(Gokhale, Larimore et al. 2012). MNT-1 cells were a generous gift of Dr. Vincent Hearing (Kushimoto, Basrur et al. 2001). Cells were maintained in DMEM supplemented with 20% AIM-V medium (Life Technologies, Carlsbad, CA), 10% FBS (heat inactivated at 65°C for 60 min), and 100 µg/ml penicillin/streptomycin at 37°C and 5% CO2. *Bloc1s5*$^{mu/mu}$, *Bloc1s6*$^{pa/pa}$, and rescued melanocytes were a gift of Dr. Michael Marks (Setty, Tenza et al. 2007, Setty, Tenza et al. 2008).

AE-iPSCs cells were obtained and cultured on human ESC-qualified Matrigel (BD Biosciences) with mTeSR-1 medium (STEMCELL Technologies) as described (Easley, Miki et al. 2012). The AE-iPSC cells were grown for a minimum of 5 days before being infected by the control and pallidin lentiviral particles for 7d in selection media as described above. After the 7d treatment the iPSCs were lifted and placed in a neural stem cell medium (DMEM F-12 +N2 supplement) containing high concentrations of EGF and FGF-2 (100ng/ml; Peprotech) and heparin (5ug/ml; Sigma) to produce cell aggregates termed EZ-spheres (Ebert, Shelley et al. 2013). To induce neuronal differentiation EZ spheres were removed from the N2/EGF/FGF/Heparin media and differentiated as follows: EZ spheres were cultured on poly-d-lysine/laminin-coated, acid etched glass coverslips for

immunofluorescence or 10 cm culture dishes for biochemical analyses in Neural Differentiation Medium containing Neurobasal, B27, Glutamax (all from Life Technologies), with 20 ng/ml GDNF (glial-derived neurotrophic factor, Peprotech) and 10 ng/ml BDNF (brain-derived neurotrophic factor, Peprotech). Medium changes on differentiating cells occurred weekly. Neuronal differentiation was confirmed by immunofluorescence microscopy using MAP2 and beta-III-tubulin as neuronal markers.

For shRNA-mediated Bloc1s5 muted and Bloc1s6 pallidin knockdowns, shRNA in a pLKO.1 vector for lentiviral infection was obtained from Open Biosystems (Pallidin - Clone ID: TRCN0000122781; Muted – Clone ID: TRCN0000128812). Control shRNA in pLKO.1 was obtained from Addgene (vector 1864). For shRNA-mediated dysbindin knockdowns, shRNA in a psiHIV-U6 vector for lentiviral infection was obtained from Genecopoeia (Dysbindin – Catalog number: HSH020444-1HIVU6). Control shRNA in a psiHIV-U6 vector was also obtained from Genecopoeia (Control catalog number – CSCHCTR001-HIVU6). SH-SY5Y cells were treated with lentiviral particles for 7 d to obtain efficient knockdown. After day 3 of lentiviral infection, cells were maintained DMEM media supplemented with 10% FBS and selected with puromycin (2ug/ml; Invitrogen) which was maintained afterwards.

cDNAS encoding EGFP-SNAP23 and SNAP525 were a gift of Dr. Gulia Baldini (Department of Biochemistry and Molecular Biology. College of Medicine at the University of Arkansas for Medical Sciences). cDNA encoding EGFP-SNAP29 was a gift from Dr. Zu-hang Sheng (NINDS, Bethesda, MA). NSF-GFP cDNA was a

gift from Dr. Phyllis Hanson (Dept. of Cell Biology and Physiology, Washington University School of Medicine, St Lois, MS). Dr. Thierry Galli provided the VAMP7-RFP cDNA (Inst. Jacques Monod, Paris, France).

SILAC labeling and mass spectrometry analysis

SH-SY5Y cells were labeled using the protocol described (Gokhale, Larimore et al. 2012, Ryder, Vistein et al. 2013). Briefly, cells were grown in DMEM media with either "light" unlabeled arginine and lysine amino acids (R0K0) or "heavy" 13C- and 15N-labeled arginine and 13C- and 15N-labeled lysine amino acids (R10K8) supplemented with 10% FBS and 100g/ml penicillin and streptomycin, and in some cases 0.2ug/l neomycin. Cells were grown for a minimum of seven passages ensuring maximum incorporation (97.5%) of the amino acids in the cellular proteins. All reagents for SILAC labeling were obtained from Dundee Cell Products. Cell lysates were prepared as described below and analyzed by mass spectrometry as described (Gokhale, Larimore et al. 2012, Ryder, Vistein et al. 2013) using the services of MS Bioworks (http://www.msbioworks.com/). Briefly, SILAC labeled samples were separated on a 4–12% Bis-Tris Novex mini-gel (Invitrogen) using the MOPS buffer system. The gel was stained with Coomassie and the lane was excised into 20 equal segments using a grid. Gel pieces were processed using a robot (ProGest, DigiLab) with the following protocol. First, slices were washed with 25 mM ammonium bicarbonate followed by acetonitrile; then they were reduced with 10mM dithiothreitol at 60°C followed by alkylation with 50 mM iodoacetamide at room temperature (RT). Samples were digested with trypsin at 37°C for 4 h and quenched with formic

acid, and the supernatant was analyzed directly without further processing. Each gel digest was analyzed by nano liquid chromatography with tandem mass spectrometry (LC/MS/MS) with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher LTQ Orbitrap Velos. Peptides were loaded on a trapping column and eluted over a 75 _m analytical column at 350nl/min; both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The 15 most abundant ions were selected for MS/MS. Data were processed through the MaxQuant software v1.0.13.13 (www.maxquant.org), which served data recalibration of MS, filtering of database search results at the 1% protein and peptide false discovery rate, and calculation of SILAC heavy:light ratios. Data were searched using a local copy of Mascot.

Immunoprecipitation

To confirm interactions between BLOC-1 subunits and NSF, we performed crosslinking in intact cells with dithiobis (succinimidylpropionate) (DSP) followed by immunoprecipitation as previously described(Zlatic, Ryder et al. 2010, Gokhale, Larimore et al. 2012, Ryder, Vistein et al. 2013). Briefly, untransfected SH-SY5Y cells or SH-SY5Y cells stably transfected with FLAG-dysbindin were placed on ice, rinsed twice with PBS, and incubated either with 10 mM DSP (Pierce), or as a vehicle control DMSO, diluted in PBS for 2 h on ice. Tris, pH 7.4, was added to the cells for 15 min to quench the DSP reaction. The cells were then rinsed twice with PBS and lysed in buffer A (150 mM NaCl, 10mM

HEPES, 1mM EGTA, and 0.1mM $MgCl_2$, pH 7.4) with 0.5% Triton X-100 and Complete anti-protease (catalog #11245200, Roche), followed by incubation for 30 min on ice. Cells were scraped from the dish, and cell homogenates were centrifuged at 16,100 *g* for 10 min. The clarified supernatant was recovered, and at least 500 μg of protein extract was applied to 30 μl Dyna magnetic beads (catalog #110.31, Invitrogen) coated with antibody, and incubated for 2 h at 4°C. In some cases, immunoprecipitations were done in the presence of the antigenic 3x-FLAG peptide (340 μM; F4799, Sigma) or antigenic GST-NSF (66 nM; H00004905-P01, Novus Biologicals) as a control. The beads were then washed 4–6 times with buffer A with 0.1% Triton X-100. Proteins were eluted from the beads with sample. Samples were resolved by SDS-PAGE and contents analyzed by immunoblot.

Sucrose density sedimentation

Control or BLOC-1 knockdown SH-SY5Y cells were rinsed twice with PBS and lysed in buffer A with 0.5% Triton X-100 supplemented with Complete antiprotease, followed by incubation for 30 min on ice. Cells were scraped from the dish, and cell homogenates were centrifuged at 16,100 *g* for 10 min. The clarified supernatant was recovered and measured for total protein content. Samples were then analyzed by immunoblot or cell lysates were resolved by sucrose sedimentation in 5–30% sucrose gradients as previously described (Gokhale, Larimore et al. 2012).

Immunofluorescence

Glass coverslips were coated with poly-D-lysine. The next day, coverslips were washed two times with cell culture water and air dried. The coverslips were then coated with laminin diluted in HBSS and placed in a 37°C tissue culture incubator for 2h. The coverslips were washed twice with cell culture water, air dried and the cells (EZ-spheres resuspended in conditioned media) were seeded onto the coverslips. The EZ spheres were grown on the coverslips for 2 weeks. At the end of the 2 weeks the cells are fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After fixation, all coverslips were washed two times in PBS and then blocked and permeabilized for 30 min at room temperature in a solution of 5%BSA, 0.1% triton –x-100 in PBS. Primary antibodies (Supplemental Table 1) were diluted in blocking solution and applied overnight at 4°C. After primary antibody incubation, coverslips were washed three times in a solution containing 0.1%triton-x100 in PBS. Coverslips were incubated for 1 hour at room temperature with fluorophore-conjugated secondary antibodies (Alexa mouse 488, Alexa rabbit 568) diluted in blocking solution. Coverslips were washed two times in blocking solution, one time in PBS, and one time in ultrapure water and then mounted on glass slides in ProLong antifade mounting medium. Coverslips were imaged by confocal microscopy as described (Zlatic, Grossniklaus et al. 2013).

S2 Drosophila and HeLa cell secretion assay

We used S2 or HeLa cells stably expressing a secretory reporter carrying a mutant FKBP protein (F36M) EGFP. Secretion was induced by AP21998 to

resolve EGFP protein aggregates in a synchronous pulse of reporter secretion. Secretion was estimated from the amount of EGFP fluorescence remaining in cells after siRNA downregulation using flow cytometry or in 96-well fluorescence plate reader (Synergy HT, Biotek). S2 cells were incubated with 20 µg of double stranded RNA (http://genomernai.dkfz.de/GenomeRNAi/) targeting syntaxin 5 as a positive control to inhibit constitutive secretion, *misfire* as a negative control (reagents DRSC10543 and AMB34062), and the *Drosophila* BLOC-1 subunits *dysbindin* (reagents DRSC10730 and DRSC35459) and *muted* (reagents DRSC36270 and DRSC36270). S2 cells were maintained for four days in the presence of double stranded RNA. Secretion pulse lasted 80 minutes. Detailed procedures were already described (Gordon, Bond et al. 2010).

Drosophila stocks, rearing, genetics, and biochemical procedures.

All fly stocks were raised throughout life at 25°C and maintained on normal food. Appropriate second and third chromosome balancers were used for all crosses. $dysb^{1}$ and UAS-Dysb were from Graeme Davis (UCSF) and UAS-NSF::FLAG was from Richard Ordway (Penn State University). w^{1118}, Elav-GAL4^{C155} and other fly strains such as balancer chromosome containing stocks are part of the Sanyal laboratory collection. Antibodies to dNSF1 and alpha-SNAP were a gift from Leo Pallanck (University of Washington, Seattle).

Intracellular recordings from muscle 6 of abdominal segment 2 or 3 of female, wandering third instar larvae were carried out in modified HL3 saline (in mM: NaCl 70, KCl 5, $CaCl_2$ 0.3, $MgCl_2$ 10, $NaHCO_3$ 10, Sucrose 115, Trehalose 5, BES [2 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid pH 7.2] 5). For all

physiological recordings, severed motor neurons were taken up into a stimulating electrode and stimulated at 1Hz for 50 s. Only those recordings where the resting membrane potential was between -60mV and -90mV and the muscle input resistance was >5MΩ were used. For acute pharmacological homeostatic challenge, semi-intact preparations were maintained with the CNS, fat bodies, and gut intact and perfused with Phillanthatoxin-433 (PhTx; Sigma), as previously described (Dickman and Davis 2009, Dickman, Tong et al. 2012). PhTx was prepared as a stock solution (4mM in DMSO) and diluted in modified HL3 to 4µM. After 10 minutes in PhTx, preparations were rinsed in modified HL3 and dissections were completed. Only recordings where the observed mEJP amplitude following PhTx incubation was less than or equal to 60% of baseline were used, indicating that the PhTx had gained access to the muscle. Microelectrodes were prepared on a magnetic glass microelectrode horizontal puller (PN-30 Narishige) to 30-70MΩ resistance and filled with 3M KCl. Signals were amplified using Axoclamp 900A, digitized using Digidata 1440A, and recorded in Clampfit 10.1. Signals were analyzed in MiniAnalysis (Synaptosoft Inc.) and Microsoft Excel. Quantal content was calculated by dividing the average EJP by the average mEJP. Correction for nonlinear summation was applied. Quantal content for each recording was calculated and then averaged across all animals for the given genotype.

Fly heads were prepared as described (van de Goor, Ramaswami et al. 1995) Briefly, approximately 100 flies per genotype were flash frozen in liquid nitrogen and decapitated. Frozen heads were collected by passing the tissue through a

microsieve in liquid nitrogen. The tissue was then ground into a powder in a mortar and pestle. The powder was combined with 100 µl lysis buffer (Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4)+ 0.5% TritonX-100 with Complete anti-protease (catalog#11245200, Roche), also frozen and ground into a powder and stored at -80°C. After thawing samples were sonicated, tissue debris removed by centrifugation and protein concentrations were determined by Bradford assay (BioRad, Villerica, CA). Proteins were resolved by SDS-PAGE on a 4-20% gel (Invitrogen) and immunoblot analysis was performed as previously described(Gokhale, Larimore et al. 2012). dNSF1 and dSNAP antibodies were gifts from Dr. Leo Pallanck(Babcock, Macleod et al. 2004).

Statistical analysis

Experimental conditions were compared using Synergy Kaleida-Graph, version 4.1.3 (Reading, PA), or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft, Vancouver, Canada).

Antibodies used

Antibody	Catalog Number	Source	Dilutions	
			IB	IF
Polyclonal Anti Muted	-	Dell 'Angelica laboratory	1:1000	-
Polyclonal Anti Pallidin	10891-AP	Proteintech Group	1:1000	-
Monoclonal Anti Pallidin (2G6)	-	Dell 'Angelica laboratory	1:500	-
Polyclonal Anti Dysbindin	HPA029616	Sigma	1:125	-
Polyclonal Anti NSF (D31C7)	3924	Cell Signaling	1:1000	-
Monoclonal Anti Actin (AC-15)	A5451	Sigma	1:1000	-
Polyclonal Anti GFP	132002	Synaptic Systems	1:2000	-
Monoclonal Anti FLAG (M2)	F3165	Sigma	1:1000	-
Polyclonal Anti FLAG	A190-102A	Bethyl	1:1000	-
Polyclonal Anti SNAP29	111 303	Synaptic Systems	1:1000	-
SNAP 25		Synaptic Systems		-
Monoclonal VAMP7		A. Peden		-
Monoclonal Anti TrFr (H84)	12-6800	Zymed	1:1000	-
Monoclonal Anti	611296	BD Biosciences	1:500	-

Tomosyn				
Monoclonal Anti Munc18	610336	BD Biosciences	1:2000	-
Polyclonal Anti Syntaxin 7	-	A. Peden	1:1000	-
Monoclonal MAP2	M1406	Sigma	-	1:1000
Polyclonal B3 Tubulin	5568	Cell signaling technologies	-	1:200

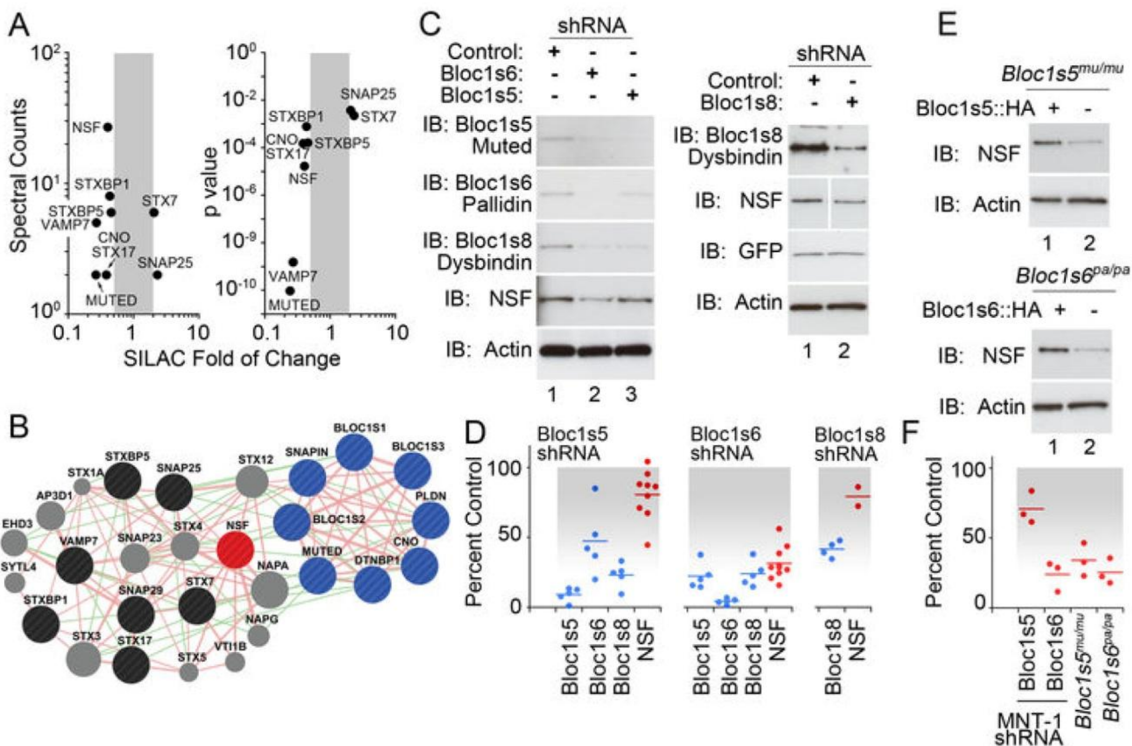


Figure 1. Fusion apparatus content is altered in BLOC-1 deficiency. A) Plots depict fusion machinery components whose content was modified by targeting the BLOC-1 subunits Bloc1s6 pallidin or Bloc1s5 muted. X axis depicts SILAC fold of change; Y axes indicate spectral counts and p value of the change. B) Interactome map of proteins modified after BLOC-1 down regulation. Pink and green lines depict predicted protein-protein and genetic interactions, respectively, as per Genemania. BLOC-1 complex subunits are in blue, NSF in red, BLOC-1 sensitive proteins in black, and proteins not identified here but predicted to be part of the interactome in gray. C) Down regulation of BLOC-1 subunits decreases NSF content in neuroblastoma cells. D) Quantification of data in C. E) We used two neuroectodermal-derived cells, MNT1 melanoma cells and immortalized melanocytes from BLOC-1 null mice. Loss of BLOC-1 reduced the content of NSF in both cells. Controls were the mutant melanocytes rescued by expression of the missing BLOC-1 gene (lane 1). F presents quantification of E. D and F all p values < 0.05.

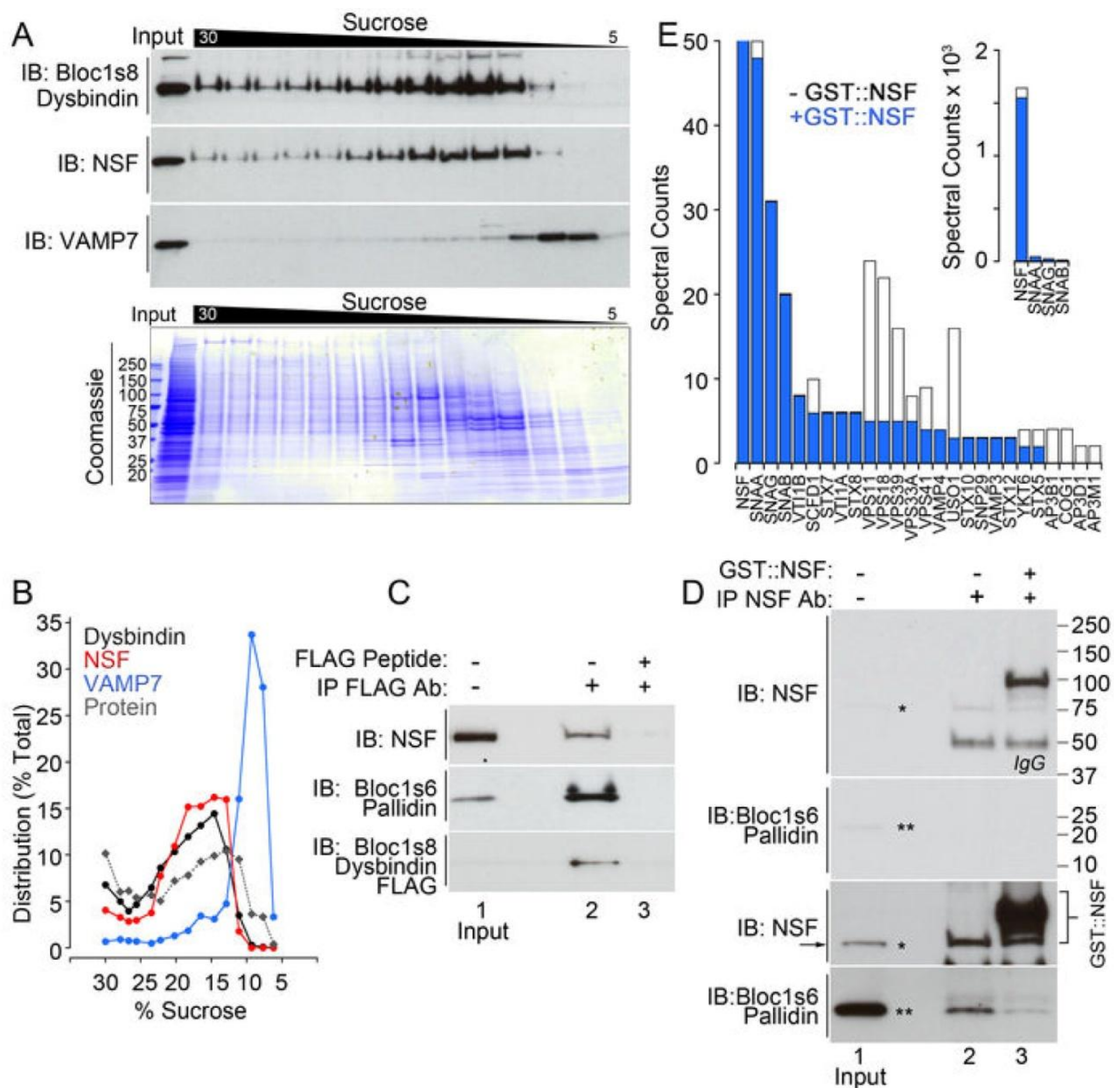


Figure 2. BLOC-1 interacts with NSF or SNAREs. A) Sucrose sedimentation of detergent-soluble cross-linked complexes from neuroblastoma cells. BLOC-1 was detected with antibodies against dysbindin. SNARE sedimentation was determined with VAMP7 antibodies. B) Relative distribution plot of data in A. C) DSP treated neuroblastoma cells expressing FLAG-dysbindin immunoprecipitate NSF with FLAG antibodies (lane 2). D) NSF antibodies immunoprecipitate Bloc1s6 pallidin (lane 2). Short and long exposures are presented. C and D lanes 3 are controls with an excess of antigen FLAG peptide or GST-NSF. E) Mass spectrometry analysis of replicate experiment as in D. White and blue bars depict lanes 2 and 3, respectively.

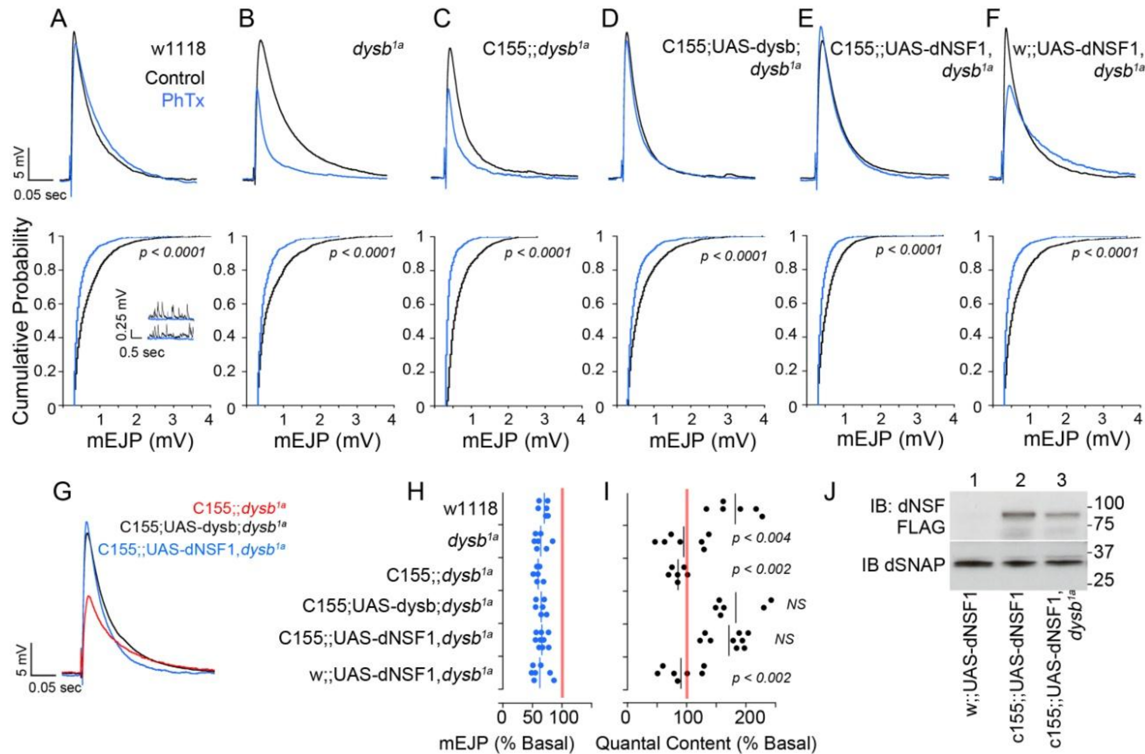
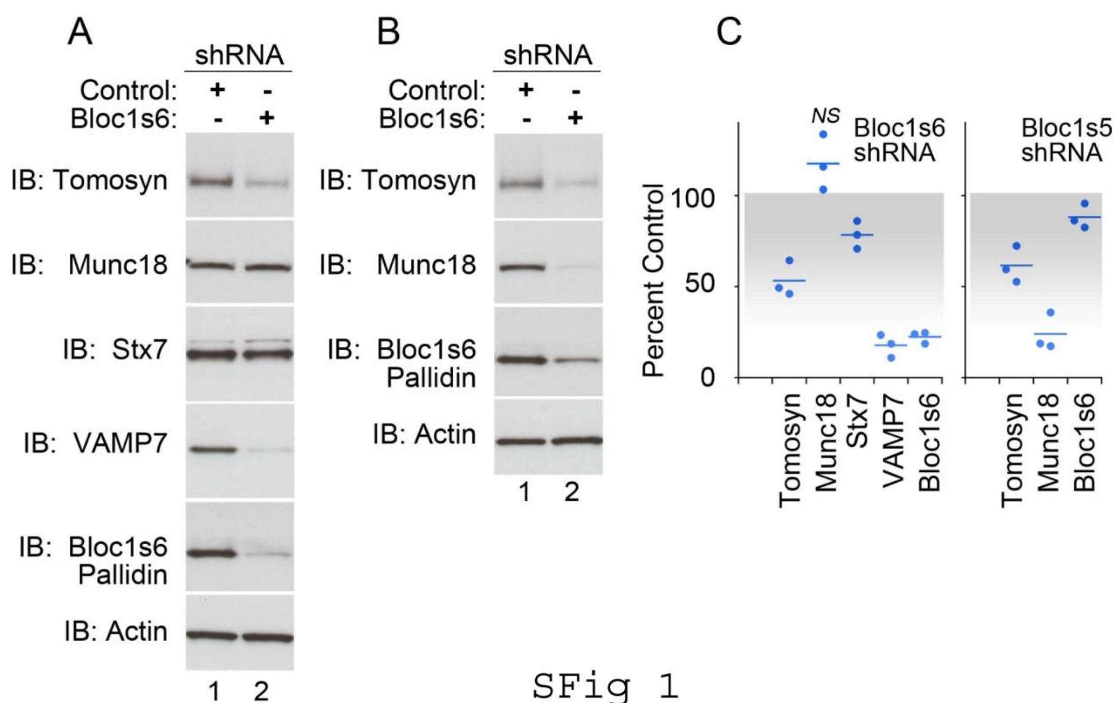
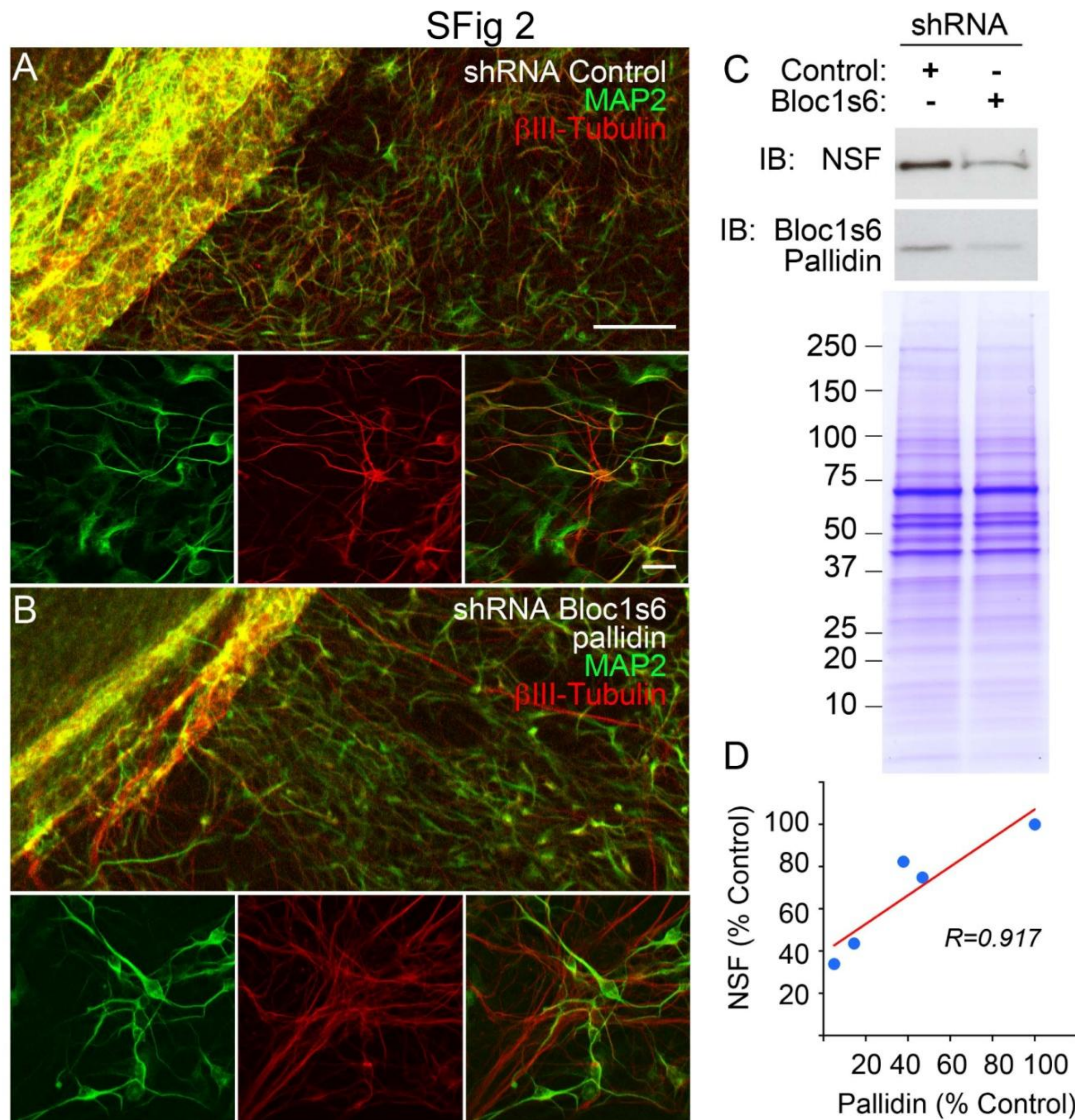


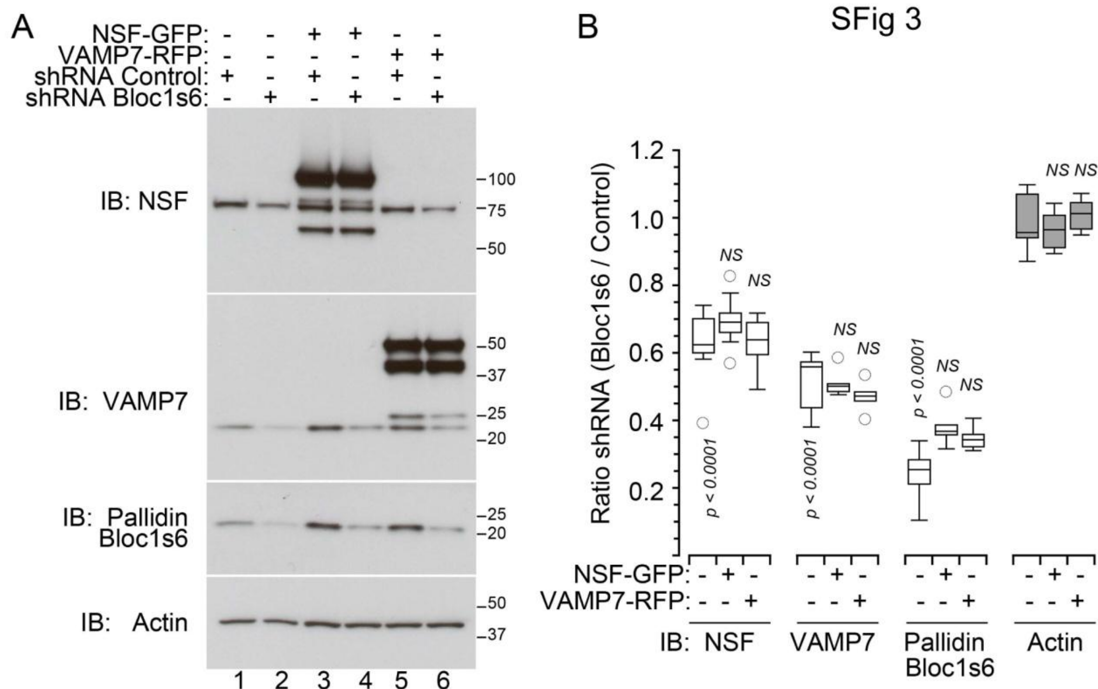
Figure 3. NSF presynaptically rescues dysbindin synaptic homeostasis defect. Representative EJP and mEJP traces without Philanthotoxin-433 (PhTx, black) and following PhTx incubation (blue). Representative mEJP traces are only shown for wild type w1118 animals. A) Wild type flies display homeostatic compensation, B-C) Mutations in *dysbindin (dysb¹)* block homeostatic compensation. D-E) *dysb¹* rescued by neuronal-specific expression of dysbindin (c155-GAL4; UAS-dysbindin; *dysb¹*) or dNSF1 (c155-GAL4;; Uas-dNSF1-FLAG, *dysb¹*). F) Uas-dNSF1 construct is insufficient to rescue synaptic homeostasis defect without c155-GAL4 driver. G) Overlay of EJP traces after PhTx presented in C-E. H) No differences across genotypes in average mEJP amplitude after PhTx. I) Presynaptic expression of dysbindin or dNSF1 display PhTx-induced homeostatic increases in quantal content equal to that of wild type. Dots represent individual animals; n>6 for all genotypes and all conditions. ANOVA-Bonferroni test. J) immunoblot demonstrating the expression of the Uas-NSF1 transgene in animals with or without the *dysb¹* mutation. dSNAP was used as a loading control.



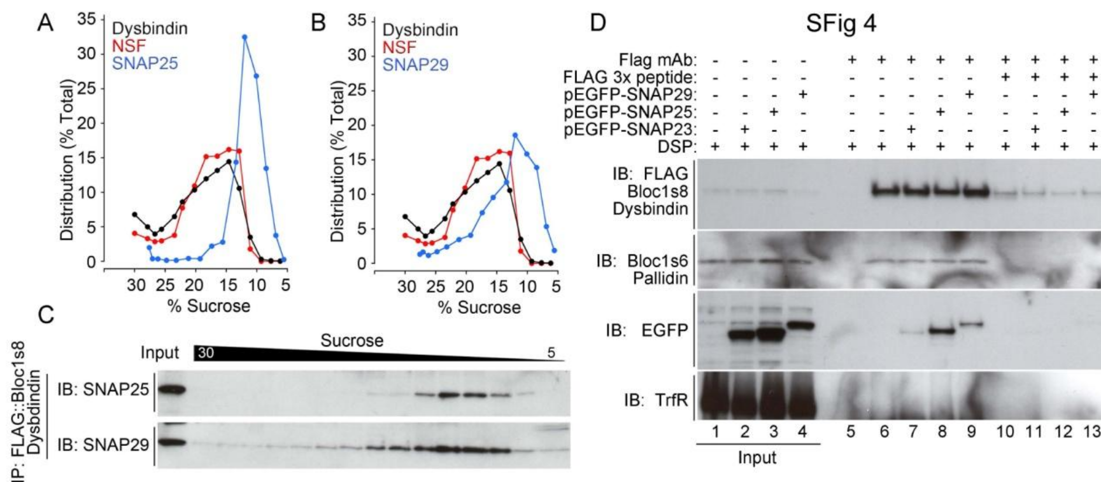
Supplementary Figure 1. Fusion apparatus content is altered in BLOC-1 deficiency. Down regulation of either BLOC-1 subunit Bloc1s6, pallidin, or Bloc1s5, muted, decreases the content of other fusion apparatus components: tomosyn, munc18, syntaxin 7 (Stx7), VAMP7 in neuroblastoma cells. C) Quantification of data in A-B. All p values < 0.05 unless otherwise indicated are not significant (NS), Wilcoxon-Mann-Whitney Rank Sum Test. Dots represent independent biological replicates.



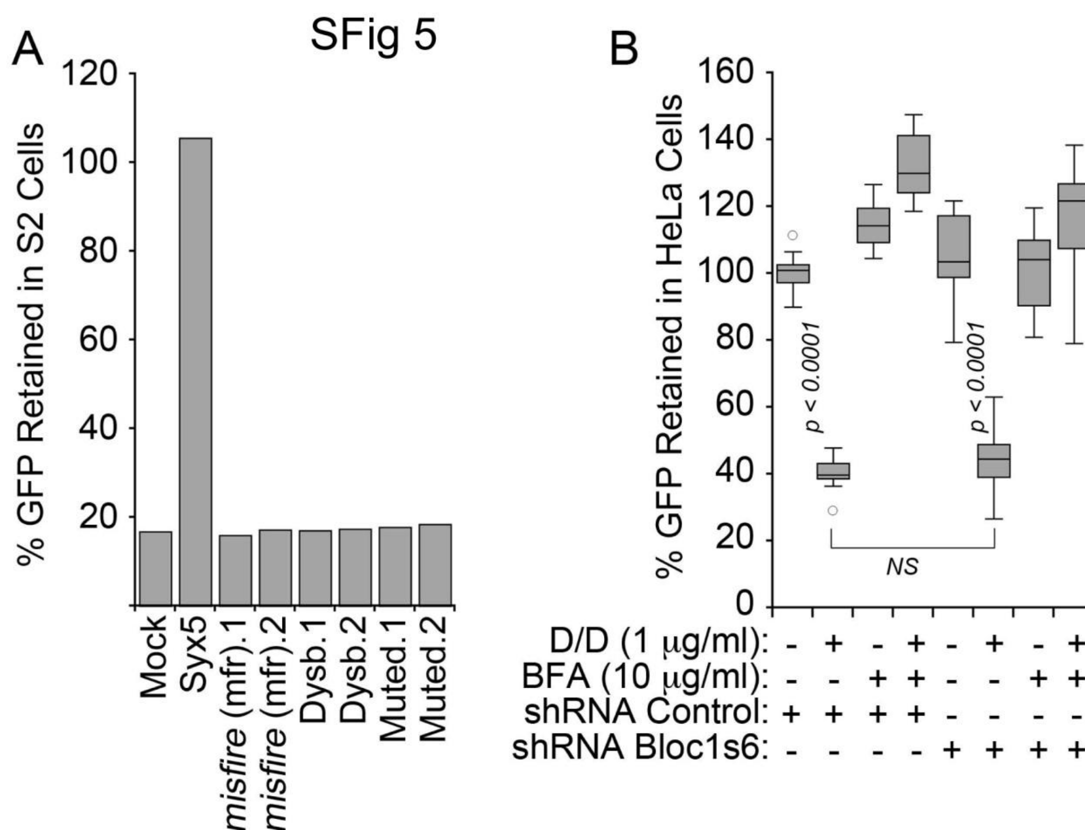
Supplementary Figure 2. Down-regulation of Bloc1s6 Pallidin in iPSC-derived Human Neurons. Human iPSC cells carrying shRNAs control or targeting Bloc1s6 pallidin were differentiated into neuronal cells for 14 days in vitro. Cells differentiation was assessed by confocal microscopy with antibodies against the neuronal markers MAP2 and beta III-tubulin (A-B). Cellular content of NSF was determined by immunoblot (C). Loading controls were performed in parallel samples resolved by SDS-PAGE and stained with Coomassie. Graph in D depicts NSF quantifications in four independent experiments.



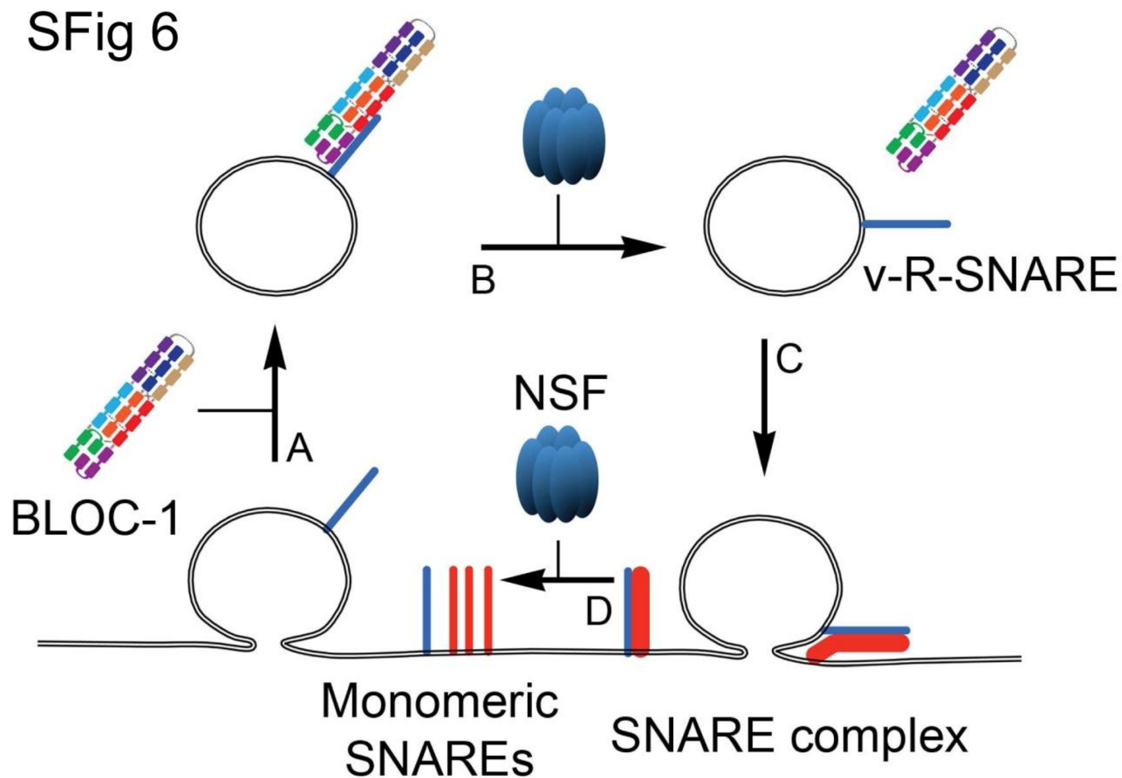
Supplementary Figure 3. NSF and VAMP7 down-regulation phenotypes are independent of each other. Neuroblastoma cells (A, lanes 1-2), cells stably either expressing NSF-GFP (A, lanes 3-4), or expressing VAMP7-RFP (A, lanes 5-6) were treated with shRNA control (odd lanes) or against Bloc1s6 pallidin (even lanes) for seven days. Cell lysates were analyzed by immunoblot with antibodies against the indicated antigens. B) Box plot depicts quantification of at five to nine determinations as a ratio of antigen content between Bloc1s6 down-regulated cells and control shRNA-treated cells. One-way ANOVA followed by Bonferroni's all pair comparisons. Significant results compare a specified antigen to actin. Non-significant results compare antigen expression either in the absence or presence of recombinant NSF or VAMP7. Neither exogenous expression of NSF nor VAMP7 rescues the NSF and VAMP7 down-regulation phenotypes induced by down-regulation of the BLOC-1 complex.



Supplementary Figure 4. Interaction of the BLOC-1 complex with SNAP23, 25 and 29. A-B) Relative distribution plots of BLOS-1 complex, SNAREs and NSF. Sucrose sedimentation of detergent soluble cross-linked complexes from neuroblastoma cells. BLOC-1 was detected with antibodies against dysbindin. SNARE sedimentation was determined with SNAP25 and SNAP29 antibodies. C) Immunoprecipitations of BLOC-1 complexes from FLAG-dysbindin expressing neuroblastoma cells. Recombinant BLOC-1 complexes resolved by sucrose sedimentation were immunoprecipitated with FLAG antibodies and immune complexes analyzed for the presence of endogenous SNAP25 and SNAP29. D) FLAG-dysbindin expressing neuroblastoma cells were transiently transfected with EGFP tagged version of SNAP23, 25 and 29. Cells were treated with DSP and BLOC-1 complexes precipitated with FLAG antibodies in the absence (lanes 5-9) or presence (lanes 10-13) of an excess FLAG peptide as antigenic competition. Immune complexes were resolved by SDS-PAGE and their composition was analyzed with antibodies against FLAG to detect recombinant Bloc1s8 dysbindin, Bloc1s6 pallidin, EGFP, and transferrin receptor (TrfR) as a control for non-specific membrane protein binding to beads.



Supplementary Figure 5. A pulse of a constitutive secretion cargo is not impaired by BLOC-1 deficiency. A) S2 *Drosophila* cells expressing a signal peptide EGFP-FKBP chimera retained in the endoplasmic reticulum were treated with mock transfection, one or two siRNA duplexes against syntaxin 5 (positive control), *misfire* (negative control), and the *Drosophila* BLOC-1 subunits *dysbindin* and *muted*. EGFP secretion was induced by disaggregation of chimeras by incubation with AP21998 and the amount of EGFP retained in cells after 80minutes of secretion was measured by flow cytometry. B) HeLa cells were treated with shRNA control or against Bloc1s6 pallidin for seven days. EGFP secretion was induced by incubation with AP21998. We measured EGFP retained in cells after 80 minutes of secretion by fluorescence plate reader in 96-well plates. Cells were incubated with brefeldin A 10 μg/ml as control to inhibit secretion. Data are depicted as box plots and compared by One-way ANOVA followed by Bonferroni's all pair comparisons (n=16 from two biological replicates).



Supplementary Figure 6. Model of BLOC-1-SNARE-NSF interactions. Step A, depicts a vesicle budding from either a donor plasma membrane or endosome in presynaptic terminals. Dysbindin-BLOC-1 complexes bind a single v-R-SNARE (blue lines) or t-Q-SNARE (red lines, binding to BLOC-1 not shown), possibly for SNARE sorting. In step B, the SNARE-BLOC-1 complex is resolved by NSF, releasing unbound BLOC-1 and fusion-competent v-R-SNARE. Step C represents a SNARE-dependent vesicle fusion event followed by resolution of a SNARE tetrahelical bundle by NSF in step D.

CHAPTER IV

DISCUSSION

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Overview

The goal of this dissertation research was to address the question of whether genetic disruptions affecting a network of closely interacting proteins, such as in a protein complex, congruently alter synaptic functions regulated by components of that network. This principle arises from the pathologies and phenotypes observed in chromosomal deletion syndromes, many of which give rise to neurodevelopmental disorders, including schizophrenia. For my dissertation research, I focused on the Biogenesis of Lysosome-Related Organelles Complex-1 (BLOC-1). Polymorphisms in the gene *DTNBP1* encoding the BLOC-1 subunit dysbindin (Bloc1s6) associate with changes in brain activation patterns (Fallgatter, Herrmann et al. 2006, Donohoe, Morris et al. 2008, Markov, Krug et al. 2009, Narr, Szeszko et al. 2009, Mechelli, Viding et al. 2010, Wolf, Jackson et al. 2011), are considered schizophrenia susceptibility risk factors (Ioannidis, Boffetta et al. 2008). Additionally, post-mortem analyses of brains of schizophrenia patients reveal a loss of dysbindin in the hippocampal formation (Talbot, Eidem et al. 2004). I use this protein complex to test my central hypothesis that:

> **BLOC-1 binding partners identified in a biochemically curated interactome converge in a defined functional pathway to regulate synaptic function.**

This prediction stems from the observations that BLOC-1 regulates the delivery of specific cargo, such as Pi4KIIa and VAMP7, to the nerve terminal (Newell-Litwa, Salazar et al. 2009, Larimore, Tornieri et al. 2011) and that synaptic function is

altered in dysbindin null animals (Dickman and Davis 2009, Ghiani, Starcevic et al. 2010). Additionally, changes in dysbindin expression regulate neurotransmitter release in mice (Chen, Feng et al. 2008), as well as deficits in NMDA receptor surface expression and signaling (Tang, Yang et al. 2009),(Karlsgodt, Robleto et al. 2011),(Jeans, Malins et al. 2011). Together, this evidence supports a greater role in BLOC-1 regulation of synaptic activity. In this work, I explore the following question in addressing my central hypothesis:

> **How do genetic modifications affecting a defined protein interaction network, in this case defined by dysbindin-containing BLOC-1, regulate properties of synaptic vesicle release and plasticity which lead to complex behaviors?**

To test my central hypothesis and answer this question, I use a biochemically curated interactome centered around the BLOC-1 subunit dysbindin to guide my investigation. This interactome identified the remaining BLOC-1 subunits as sensitive to dysbindin perturbation. I use the *Drosophila* neuromuscular junction as a model synapse for testing the role of these interactions on regulation of multiple synaptic functions. Additionally, I employ the *Drosophila* olfactory system to test the ability of these interactions to regulate a circuit-based learning behavior (short-term olfactory habituation). This approach led to the following findings concerning the interaction between BLOC-1 subunits, described in Chapter II:

1) Orthologous BLOC-1 subunits in *Drosophila melanogaster* coprecipitate with the BLOC-1 subunit dysbindin. The identification of all eight BLOC-1 orthologues by mass spectrometry analysis confirms the assembly and

presence of an orthologous octameric BLOC-1 complex in *Drosophila* neurons.

2) BLOC-1 acts presynaptically to regulate quantal content and morphology at the *Drosophila* neuromuscular junction. Loss of Blos1 is a dominant, gain-of-function phenotype, which can be suppressed in the absence of dysbindin.
3) Dysbindin and Blos1 are necessary for the function of multiple synaptic vesicle pools. Unlike quantal content and NMJ morphology, function of synaptic vesicle pools behaves in a recessive, partial loss-of-function manner.
4) Dysbindin and Blos1 are required for olfactory short-term habituation in *Drosophila*. In contrast to the above traits, STH follows a strict recessive inheritance pattern.

Additionally, the dysbindin-centric interactome identified synaptic vesicle fusion machinery as BLOC-1 sensitive factors, the interactions of which I tested and drew the following conclusions towards in Chapter III:

1) Fusion apparatus content is altered in BLOC-1 deficiency. SILAC analysis following downregulation of BLOC-1 subunits in SH-Sy5Y cells revealed concurrent downregulation of synaptic vesicle fusion machinery, including NSF.
2) BLOC-1 interacts with NSF or SNAREs, but not simultaneously. Biochemical analysis confirmed BLOC-1 interaction with both NSF and

SNAREs. However, mass spectrometry analysis revealed that NSF bound either SNAREs or BLOC-1 in cells in culture.

3) NSF acts downstream of dysbindin to presynaptically rescue the dysbindin synaptic homeostasis defect.

These findings support three novel hypotheses, which I will discuss in turn below. First, phenotypes arising from loss-of-function mutations to members of the BLOC-1 complex are governed by the dosage balance hypothesis. This hypothesis was first formulated following observations that changes in chromosome number had much more dramatic effects on organismal phenotypes than did whole-genome changes (Blakeslee, Belling et al. 1920, Birchler and Veitia 2007). In the earliest studies of the dosage balance hypothesis, it was readily established that sex determination is tightly regulated by the ratio between the two sex chromosomes, in plants as well as animals (Dellaporta and Calderon-Urrea 1993, Birchler and Veitia 2007). Until recently, interpretations of the dosage balance hypothesis have largely postulated that phenotypes arise from a misregulation of the ratio of *regulatory* genes in cases of aneuploidy (Birchler, Bhadra et al. 2001, Birchler, Riddle et al. 2005, Birchler and Veitia 2007, Veitia, Bottani et al. 2008). This was well supported by the identification of modifiers of the *white* eye color gene in *Drosophila*, which fell into one of two major categories based on molecular basis: signal transduction, or transcription/chromatin binding factors (Birchler and Veitia 2007, Veitia, Bottani et al. 2008). While factors which form transcriptional regulatory complexes are indeed overrepresented in cases of haploinsufficiencies, the dosage balance hypothesis has since been predicted to

govern the activity of other protein-protein complexes as well (Birchler and Veitia 2007). Importantly, however, this work is the first to observe the dosage balance hypothesis in the membrane-trafficking field, which traditionally predicts that loss-of-function to subunits within a complex would have identical phenotypes. In fact, the ability for loss-of-function mutations to subunits within a complex to phenocopy each other is often noted as a defining characteristic of what constitutes a subunit of a complex. Thus, this study highlights an outdated conception of the interactions of the subunits of a protein complex within the membrane-trafficking field to which BLOC-1 belongs. Further, the novelty of my findings lie not in the observation of the dosage dependent effect, as this is an established genetic phenomenon, but rather in the observation of this effect with specific regards to BLOC-1, the regulation of synaptic transmission, and the significance of these interactions in understanding complex neuropsychiatric and neurodevelopmental disorders.

The dosage balance hypothesis predicts that there is a relationship between the number of interactions that a component has, and the degree of variation that occurs when that component is either under- or over-expressed. In fact, an inverse relationship between the degree of connectivity of a gene or its encoded protein within a network and the genetic variation associated to that locus has been well-described using quantitative genetics in yeast, where the higher the connectivity of a component within a network, the lesser the extent of variation associated with gene expression (Lemos, Meiklejohn et al. 2004). The high conservation of genes within tightly connected networks suggests that variation

of these genes would have a greater impact on the organism than variation of less connected, peripheral network components (Lemos, Meiklejohn et al. 2004, Veitia, Bottani et al. 2008). Additionally, the dosage balance hypothesis predicts that loss-of-function mutations in two or more genetic loci encoding polypeptides belonging to the same protein interaction network do not follow a simple genetics pattern of phenocopying each other or confering additive functional consequences; rather, phenotypes are governed by differential sensitivities to interactive and stoichiometric genetic dosages of complex constituents (Birchler and Veitia 2007). Second, genotype-to-phenotype correlations observed in a trait following a gene pair analysis can better, although not precisely, predict how other traits may respond. Finally, polypeptides that are associated with a disease, form a biochemical network, and are all sensitive to genetic perturbation of a common network constituent, converge in a defined functional pathway where endophenotypes can be assessed.

In the following sections, I will first briefly summarize the findings that led to these hypotheses. I will then discuss each of these hypotheses in detail and use them to explore the implications for our understanding of BLOC-1 in synaptic functions, as well as expand the implications for these findings with respect to the larger context of complex genetic disorders, such as copy number variations associated to human neurodevelopmental diseases. In doing so, I will briefly discuss future directions for this work as well as provide an outlook on how *Drosophila* can provide a viable model system for understanding complex neurological disorders.

Summary of Results

In this research, I used the *Drosophila* NMJ to test synaptic properties regulated by loss-of-function alleles affecting the BLOC-1 subunits *dysbindin* and *blos1*, in isolation or in combination. Loss-of-function alleles affecting Blos1 alone were dominant, gain-of-function mutations resulting in increased mEJP amplitudes and elaborated synaptic morphology. *Blos1* mutation gain-of-function dominant phenotypes, however, were suppressed by single copy loss of *dysbindin*. From these findings, I concluded that gene dosage reductions in two or more genetic loci encoding polypeptides belonging to the same membrane-trafficking protein interaction network follow the dosage dependent hypothesis rather than confer an additional or equal loss of function phenotype. Additionally, when the Blos1 loss-of-function allele was expressed in trans with the dysbindin loss-of-function allele, acute philanthotoxin-induced synaptic homeostasis was impaired to the same extent as in double copy loss of *dysbindin*. Animals carrying the transheterozygotic mutations to *blos1* and *dysbindin* also had defects in the synaptic vesicle reserve pool, as observed by rapid signal depletion during high frequency stimulation in the presence of bafilomycin A1. This phenotype was also observed in blos1 null animals, but not in $dysb^1$ animals. However, when combinations of these alleles were tested in a behavioral paradigm, olfactory short-term habituation, double copy loss of both *blos1* and *dysb* animals demonstrated impaired phenotypes. Additionally, the transheterozygotic animals were also impaired in STH, but single copy loss of either *blos1* or *dysbindin* had no effect. Thus, hierarchical clustering analysis demonstrated that the genetic

patterns underlying these three phenotypes clustered together better than any of these grouped with either the quantal content analysis or the assessment of synaptic morphology. Taken together, these findings support the conclusion that genotype-to-phenotype correlations observed in a trait following a gene pair analysis such as the transheterozygotic condition, better, although not precisely, predict how other traits may respond. Finally, I used SILAC labeling to identify cellular factors that are sensitive to BLOC-1 downregulation by shRNA targeted against dysbindin or muted in SH-SY5Y neuroblastoma cells. Proteins identified by mass spectrometry analysis included the synaptic vesicle fusion machinery components SNAP25, Syntaxin family members STX1A, STX12, STX4, STX5, STX 3, and NSF. Changes in cellular content of these factors in response to BLOC-1 downregulation were confirmed in independent shRNA experiments in human iPSCs and SH-SY5Y cells in culture, and interactions between SNARE proteins or NSF and BLOC-1 were confirmed by immunoprecipitation. Identification of BLOC-1 sensitive proteins directly involved in synaptic vesicle regulation allowed me to test the hypothesis that these factors converge in a BLOC-1-regulated pathway- namely, that of acute, philanthotoxin-induced synaptic homeostasis. NSF expressed presynaptically in $dysb^1$ animals rescued their loss of synaptic homeostatic compensation ability, suggesting that NSF resides downstream of dysbindin-BLOC-1 to regulate this pathway. This finding supports that polypeptides that form a biochemical network and are all sensitive to genetic perturbation of a common network constituent, converge in a defined functional pathway.

Hypothesis 1: Phenotypes arising from loss-of-function mutations to members of the BLOC-1 complex are governed by the dosage balance hypothesis.

The simplest predictions regarding loss-of-function mutations to components within a protein complex or even within a larger network are that: first, loss-of-function mutations to separate members of the complex would phenocopy each other, or second, that combinations of loss-of-function mutations to multiple components would exacerbate the extent of a phenotype. However, the dosage balance hypothesis postulates that stoichiometry between components rather than the total number of components governs the phenotypic outcome. This first hypothesis predicts just this- that loss-of-function mutations in two or more genetic loci encoding polypeptides belonging to BLOC-1 do not confer equivalent or additional loss of function consequences. Rather, phenotypes are governed by differential sensitivities to genetic dosages of BLOC-1 constituents. This hypothesis further predicts that mutations which affect the different genes encoding protein complex subunits may yield disparate phenotypes as well as mechanisms of inheritance.

In the discussion of Chapter II, I propose that the observed dominant and recessive phenotypes following combinations of BLOC-1 loss of function mutations support this dosage balance hypothesis. The data I present in Chapter II strongly supports the mechanisms of inheritance aspect of this hypothesis, which is best evidenced by the recessive nature of loss-of-function mutations on olfactory short-term habituation compared to the dominant gain of function

effect of *Blos1* mutations on quantal content and branching phenotypes. Further, I propose that the observed phenotypes are ultimately a result of remnants of BLOC-1, and I suggest three non-mutually exclusive possibilities:

1) Mutations to different BLOC-1 subunits may result in similar reductions of BLOC-1 content and activity.

2) 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.

3) Loss-of-function mutations affecting BLOC-1 subunits may lead to gain-of-function remnants.

I discuss these possibilities in detail in Chapter II, and thus will not further elaborate on them here. However, there is an additional non-mutually exclusive possibility that arises from this hypothesis that I have not yet discussed but warrants contemplation, as it is equally as likely as any of the aforementioned:

4) Loss-of-function mutations affecting BLOC-1 subunits may lead to BLOC-1 partial loss-of-function subcomplexes.

These hypotheses are summarized in Figure 1, along with the phenotypes that may be attributed to each possibility. The gene dosage hypothesis predicts that following loss-of-function mutations to complex subunits, components of the complex remain within the cell. In Hypothesis 3, I suggest that the residual complex elements assemble into gain-of-function remnants. However,

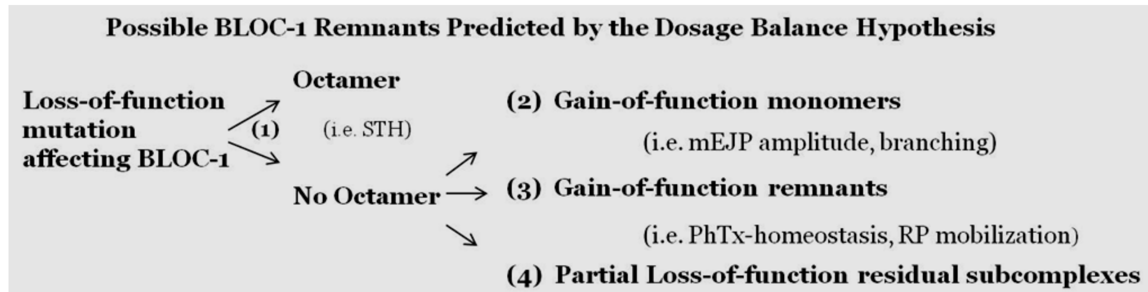

Figure 1. Possible BLOC-1 Remnants Predicted by the Dosage Balance Hypothesis. Flowchart summarizing the four non-mutually exclusive possibilities of the effects of loss-of-function mutations affecting BLOC-1 as derived from the dosage balance hypothesis. Phenoypes studies in Chapter II are displayed between possible remnants from which they may arise.

Hypothesis 4 predicts that the residual elements represent loss-of-function pieces of the complex. This interpretation predicts that the specific functions of BLOC-1 depend not necessarily on the entire octamer, but are carried out by subdomains of the assembled complex. Several lines of evidence support this possibility. First, loss-of-function mutations to members of a protein complex result in downregulation of the remaining subunits. This is certainly the case for BLOC-1, as evidenced by dramatic reductions in dysbindin protein levels in pallid mice brain extracts (Ghiani, Starcevic et al. 2010). It is currently believed that the residual expression levels represent unstable monomeric forms of the remaining subunits, but the participation of these remaining subunits outside the traditional complex roles has not yet been studied in detail. Second, two stable, heterotrimeric subcomplexes of BLOC-1 were recently defined in vitro, containing Pallidin-Cappuccino-BLOS1 and Dysbindin-Snapin-BLOS2 (Lee, Nemecek et al. 2012). While no specific function has yet to be attributed to these subcomplexes, the authors speculate that the two subcomplexes could comprise flexible domains within the octamer to allow BLOC-1 to interact with multiple

binding partners simultaneously. Finally, while specific interactions between subunits within the complex have been defined (Starcevic and Dell'Angelica 2004, Nazarian, Starcevic et al. 2006), and many binding partners outside of the complex have been identified (Di Pietro, Falcón-Pérez et al. 2006, Nazarian, Starcevic et al. 2006, Salazar, Craige et al. 2006, Ghiani, Starcevic et al. 2009, Rodriguez-Fernandez and Dell'Angelica 2009, Salazar, Zlatic et al. 2009, Larimore, Tornieri et al. 2011, Gokhale, Larimore et al. 2012), it is still unknown how the various subunits differentially engage in these binding events. Unfortunately, the tools in the field, specifically the lack of antibodies against each of the subunits, in mammals and even more so in flies, have limited the extent to which BLOC-1 architecture and specific binding have been able to be studied, such that it is currently unknown which BLOC-1 subunits mediate binding with the various interactors.

Taken together, this suggests the possibility that BLOC-1 itself is segregated into distinct functional domains, which are defined by extra-complex interactions and mediate specific functions. Thus, loss-of-function mutations affecting BLOC-1 may lead to differential downregulation of subdomains containing subcomplexes. The ultimate result in this instance would be deficits in one BLOC-1 mediated function but retention of another, and would be determined by the initial subunit affected. Analysis of BLOC-1-mediated vesicle pool engagement in Chapter II supports this final possibility. Here, we see that dysbindin mutants are defective in philanthotoxin-induced homeostatic response, while blos1 mutants are unaffected. Conversely, blos1 mutants display early vesicle depletion during high

frequency stimulation while dysbindin mutants are unaffected. It would follow from this hypothesis that dysbindin-containing subdomains and blos1-containing subdomains mediate different BLOC-1 cellular engagements. However, transheterozygotes possessing single copy loss of each dysbindin and blos1 display deficits in both phenotypes. Thus, it would also follow that the subdomains described must overlap. If this is the case, we would expect there to be a phenotype that is affected by all three conditions and requires synaptic vesicle sorting or recruitment similar to that required by homeostatic compensation or reserve pool engagement. In fact, this is exactly what we observe in the case of olfactory short-term habituation, which hierarchical clustering analysis groups with homeostatic compensation and reserve pool mechanisms.

The loss-of-function of various subdomains may occur due to altered interactions with binding partners at various stages of the synaptic vesicle lifecycle. Thus, BLOC-1 loss of function mutations that specifically disrupt binding between BLOC-1 and a known interactor, such as AP-3, would only perturb cellular functions dependent on this interaction, but not cellular functions that are independent of this interaction (Figure 2). This is evidenced by the bafilomycin assay reported in Chapter II. During low frequency stimulation, synaptic transmission is sustained through recycling of vesicles at the level of the readily releasable pool, with minimal recruitment from the reserve pool of vesicles. BLOC-1 is likely not to be involved in this process, as the mutations studied here had no effect on the rate of vesicle depletion under low frequency stimulation. Lack of involvement of BLOC-1 on this pool of vesicles is further supported in

Chapter III, as constitutive secretion in *Drosophila* S2 cells is unaffected by BLOC-1 downregulation (Supplementary Figure 5). Alternatively, it could be that the remnants that are remaining in these mutations have no effect on this pool of vesicles. However, at high frequency stimulation, transmission is sustained by the engagement of the reserve pool of vesicles. During high frequency stimulation, vesicle recycling to replenish the depleting reserve pool occurs through activity-dependent bulk endocytosis. Through this process, deep invaginations of the plasma membrane form, which can then sort vesicles directly via clathrin-dynamin mediated budding steps (Figure 2, Pathway 1). Alternatively, these plasma membrane invaginations can give rise to endosomal compartments, where cargo can be sorted into synaptic vesicles for vesicle pool replenishment (Figure 2, Pathway 2). The generation of synaptic vesicles from activity-dependent bulk endosomes is mediated by AP-3 and AP-1 in parallel pathways, whereby distinct vesicle populations are distinguished by vSNARE-binding specificity. Thus, BLOC-1 loss of function remnants may be interfering with the cell's ability to generate or replenish the reserve pool and maintain synaptic neurotransmission through BLOC-1-AP-3 dependent sorting mechanisms at activity-dependent bulk endosomes (Figure 2). The engagement of the reserve pool of vesicles during high frequency stimulation, however, is inherently different from presynaptic compensation mechanisms that are required during acute synaptic homeostasis. This is evident by the divergent genotype-to-phenotype correlations observed in these studies. Blockade of the postsynaptic receptors is thought to trigger the release of a retrograde signal that leads to acute synaptic homeostatic compensation through two mechanisms: 1) an increase in

releasable vesicle pool size, and 2) an increased rate of vesicle turnover 16129401. This process does not require the replenishment of the reserve pool of vesicles ; thus it is not dependent on the BLOC-1-AP-3 interaction.

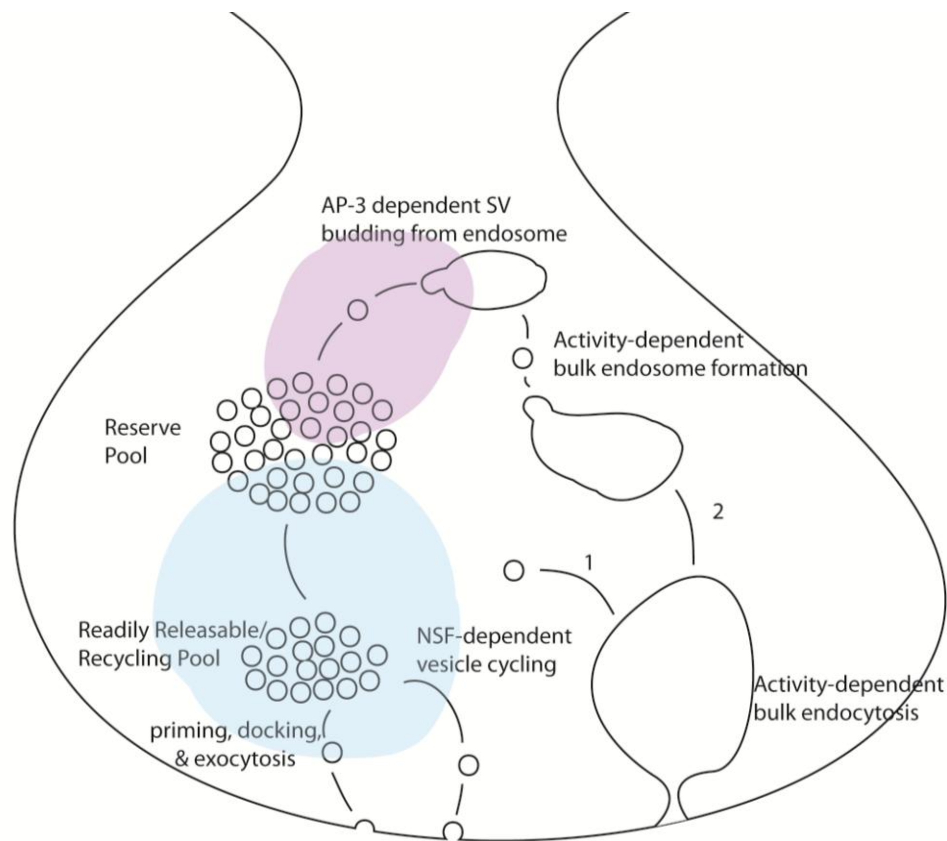

Figure 2. Cellular processes within the presynaptic compartment involving BLOC-1 are potentially governed by distinct BLOC-1 molecular interactions. Blue region represents BLOC-1 mediated pathway that is sensitive to residual BLOC-1 following dysbindin loss of function; purple region represents BLOC-1 mediated pathway that is sensitive to residual BLOC-1 follow blos1 loss of function. Activity-dependent bulk endocytosis following high frequency stimulation gives rise to two vesicle budding pathways. Pathway 1 involves direct clathrin-dynamin vesicle budding from the plasma-membrane connected invaginations. In pathway 2, these invaginations give rise to activity dependent bulk endosomes which in turn bud synaptic vesicles via AP-1 (not shown) or AP-3 dependent sorting mechanisms.

Rather, separate dysbindin-BLOC-1 interactions may be responsible for facilitating the increase in quantal content during homeostatic compensation through three processes related to the aforementioned mechanisms. As suggested by the data in Chapter III, NSF may have a role in the vesicle cycle that is downstream of the role of BLOC-1, such that the cell can compensate for the absence of BLOC-1 with the addition of NSF but NSF does not replace a function of BLOC-1. BLOC-1 may designate a subset of vesicles within the readily releasable pool such that at least two opportunities arise for the interaction of NSF and BLOC-1 to regulate releasable vesicle properties. First, BLOC-1 decorated vesicles in the readily releasable pool may need to shed v-SNARE-bound BLOC-1 before vesicle tethering can occur. NSF may resolve BLOC-1-SNARE interactions to render v-SNAREs competent for interactions with tethering factors (Figure 3, Top panel, A. SNAREs not depicted for simplicity). Alternatively, following the resolution of tetrahelical SNARE complexes after fusion events, NSF may hand newly freed, unbound SNAREs over to BLOC-1 for trafficking back to the readily releasable pool (Figure 3, Top panel, B). BLOC-1 may then determine a return route from the plasma membrane in vesicle recycling, targeting rapidly recycling vesicles from the active zone to a readily releasable pool, likely through interactions with SNAREs (Figure 3, Top panel, C). In the absence of BLOC-1, NSF may be available to interact with coat complexes for which it has a lower affinity to similarly prepare v-SNAREs and vesicles for docking, and is thus able to increase the number of released vesicles in this manner (Fig 3, bottom panel, A). Further, vesicles of the readily releasable pool do not get labeled with BLOC-1 (Figure 3, Bottom panel, B). Additionally,

the ability of NSF to rescue the cell's potential for homeostatic compensation in the absence of dysbindin-BLOC-1 supports a need for increased vesicle turnover rates and direct recycling during synaptic homeostasis (Figure 3, Bottom panel, C.).

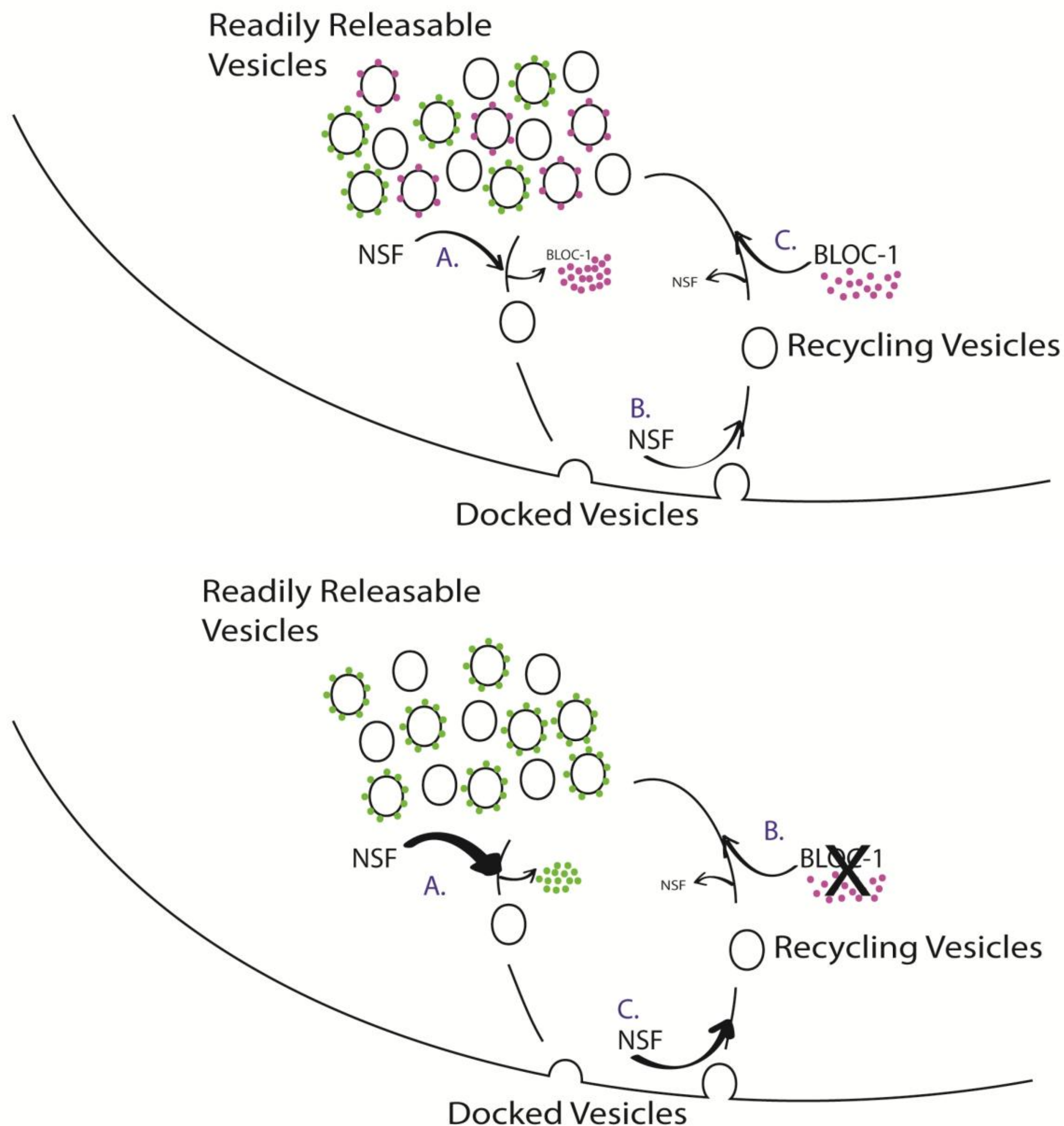

Figure 3. Potential steps of NSF and BLOC-1 involvement and interaction during fast vesicle cycling at the plasma membrane. *TOP PANEL*: (A): BLOC-1 (purple) decorates a subset of vesicles in the readily releasable pool through interactions with v-SNAREs (not shown for simplicity) and is shed in an NSF-dependent step to make v-SNAREs available for vesicle tethering and fusion. 'Other' coat proteins are depicted in green. (B). NSF resolves tetrahelical SNARE bundles, and acts to 'hand-off' freed SNARE-containing vesicles to BLOC-1. (C). BLOC-1 may target a subset of recycling vesicles to the readily releasable pool. *BOTTOM PANEL:* Addition of NSF compensates for loss of BLOC-1. (A) In the absence of BLOC-1, NSF sheds 'other' coat proteins (green) for which it has lower affinity to increase the number of released vesicles. (B) Loss of BLOC-1 decreases number of BLOC-1 decorated vesicles at the RRP. (C) Increased NSF supports greater ability for vesicle turnover.

Hypothesis 2: Genotype-to-phenotype correlations observed in a trait following a gene pair analysis can better, although not precisely, predict how other traits may respond.

The hierarchical clustering analysis presented in Chapter II highlights both the power and limitations of predicting phenotypes based on a single genotype, as well as in the absence of knowledge of how a particular gene assembles into a protein interaction network. The data behind this analysis demonstrates that the correlations between genotype and phenotype cannot be predicted based on a single allele when the gene in question participates in an interconnected biochemical and genetic pathway. In these cases, using a gene pair analysis provides greater insight into the predicted phenotype, the complexity of the genes involved, and the potentially divergent mechanisms of inheritance than can be drawn from information concerning a single allele.

In Chapter I Section 4, I introduced the advantages of using *Drosophila* as a model system by citing the Fragile X Syndrome success story. One key aspect of these studies is the fact that this syndrome is purely monogenic, and thus there is a perfect genotype-to-phenotype correlation, such that we know that loss of FMR1 *causes* FXS. In the case of schizophrenia, and other polygenic disorders, our approach need not be entirely different. Similar to the work on FXS, I predict that using an endophenotype to study chromosomal deletion syndromes and neurodevelopmental disorders in a model system will prove successful. However, in studying these disorders, it is not sufficient to predict outcomes simply based on the loss of a single gene associated to the disorder. Rather, endophenotypes in

model systems, such as *Drosophila*, are more successfully predicted when we consider two or more genes involved. While this may seem intuitive, most experimental 'models' of schizophrenia and other polygenic neurodevelopmental disorders are still pushing the analysis of a single gene or a single gene product on neurodevelopmental, synaptic, and behavioral phenotypes. Additionally, more than one endophenotype for a disorder may need to be identified and characterized, as demonstrated by the segregation of the observed phenotypes in Chapter II into two distinct genotype-to-phenotype clusters. Monogenic disorders, such as FXS, provide support for the belief that the better the genotype-to-phenotype correlation, the more predictable genetic or pharmacological manipulations to the system can be. Through my dissertation research, I propose we move away from monogenic analyses as we move forward in understanding these complex genetic diseases, which span a large spectrum of phenotypes and the number of genes involved seems to be continually expanding.

Hypothesis 3: Polypeptides that are associated with a disease, form a biochemical network, and are all sensitive to genetic perturbation of a common network constituent, converge in a defined functional pathway where endophenotypes can be assessed.

In Chapter I Section 1: Neurodevelopmental Disorders: Mechanisms and Boundary Definitions from Genomes, Interactomes, and Proteomes, I introduce the notion that biochemically curated interactomes could be used to guide investigation of complex genetic disorders. I present an argument for putting attention to the degree of phenotypic and genotypic overlap between chromosomal deletions associated with NDDs, highlighting that if we expand our definition of overlapping risk factors to include putative binding partners, the genetic overlap between associated genes jumps from 1 in 700 to an astounding 147 in 700 (Cristino, Williams et al. 2013). If we consider that there are currently nearly 9000 polymorphisms associated to over 1000 genes that we have identified as associated with risk of schizophrenia(Allen, Bagade et al. 2008), the question of where to begin in unraveling the contribution of each of these factors is overwhelming. Thus, the field of schizophrenia genetics necessitates a high-throughput genetic screening method that focuses on the synapse. Here, I present evidence for the effective use of such a method and discuss the possibilities my research presents to the field at large.

Chapter III serves as proof of principle for this argument. I introduce the derived interactome for dysbindin, confirm the observed interactions with fusion machinery biochemically, and test the hypothesis that these factors converge in a

functional pathway. The ability of NSF to rescue acute synaptic homeostasis abilities in dysbindin mutants suggests that NSF resides downstream of dysbindin-BLOC-1 in regulating this process, as described above. Further, in Chapter II, I demonstrate that the removal of a single copy of blos1 in the dysb1/+ animal conveys a deficit in homeostatic compensation, while single copy loss of either allele alone bears no significance. Based on these results, I propose that the philanthoxin-induced synaptic homeostasis can serve as one endophenotype for studying schizophrenia genetics in *Drosophila*, and that this assay may be a viable, high-throughput screening method for:

1) Assessing genetic interactions within a complex or interactome that will convey loss-of-function phenotypes, as is the case with dysbindin-blos1, and

2) Identifying steps and factors downstream of the 'guilty' genes that can rescue the endophenotype.

By using a biochemically curated and confirmed interactome to investigate endophenotypes, such as synaptic homeostasis, we will be able to identify combinations of genetic variations that may convey greater risk of disease than others. Additionally, by using this approach to identify factors that can rescue the endophenotype, we will be able to generate a robust understanding of the factors and pathways involved, as was the case with the study of *fmr1* and *dfmr1* as described in Chapter 1. In following this example, we may be able to use these combined approaches to generate a new age of targeted therapeutics.

CHAPTER V

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