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Review

Drosophila models of early onset cognitive disorders and their clinical applications

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ABSTRACT

The number of genes known to cause human monogenic diseases is increasing rapidly. For the extremely large, genetically and phenotypically heterogeneous group of intellectual disability (ID) disorders, more than 600 causative genes have been identified to date. However, knowledge about the molecular mechanisms and networks disrupted by these genetic aberrations is lagging behind. The fruit fly *Drosophila* has emerged as a powerful model organism to close this knowledge gap. This review summarizes recent achievements that have been made in this model and envisions its future contribution to our understanding of ID genetics and neuropathology. The available resources and efficiency of *Drosophila* place it in a position to tackle the main challenges in the field: mapping functional modules of ID genes to provide conceptually novel insights into the genetic control of cognition, tailored functional studies to improve 'next-generation' diagnostics, and identification of reversible ID phenotypes and medication. *Drosophila's* behavioral repertoire and powerful genetics also open up perspectives for modeling genetically complex forms of ID and neuropsychiatric disorders, which overlap in their genetic etiologies. In conclusion, *Drosophila* provides many opportunities to advance future medical genomics of early onset cognitive disorders.

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1. Introduction

The human brain is one of nature's most sophisticated structures. During brain development, billions of neurons are born and organized into complex networks. Post development, the brain maintains structural and functional plasticity, which allows us to learn, memorize and to adapt our behavior accordingly. Genetic defects that compromise brain development, function, and plasticity can cause cognitive disorders such as intellectual disability (ID).

ID, previously referred to as mental retardation, is a collective term for a group of phenotypically and genetically heterogeneous diseases that share impaired cognitive function as a common hallmark (van Bokhoven, 2011). ID is defined by an IQ lower than 70, presenting with deficits in adaptive behavior (e.g. self-care, social and interpersonal skills) at an age of onset before 18 years (American Psychiatric Association, 1994). This definition distinguishes ID from late-onset neurodegenerative disorders. ID affects as much as 2% of the population and, because of the high frequency and the need for lifelong care, the total health care expenditures far exceed those of other mental handicaps (Ropers and Hamel, 2005). ID is thus a major unsolved medical and socioeconomic challenge for society (Ropers, 2008).

ID is often distinguished into non-syndromic and syndromic forms. In syndromic ID, patients present with clinical features in addition to ID, such as behavioral abnormalities, dysmorphisms, and metabolic defects. ID can also be classified by severity, ranging from mild (IQ 50–69) and moderate ID (IQ 35–49) to severe (IQ 20–34) and profound ID (IQ <20) (American Psychiatric Association, 1994). Clinical and genetic aspects of ID have been extensively reviewed elsewhere (Ellison et al., 2013; Mefford et al., 2012; Ropers, 2008; van Bokhoven, 2011). To date, more than 600 genes have been identified that cause ID when mutated (our unpublished literature survey), yet the majority of ID genes still await discovery (van Bokhoven, 2011).

Because of the genetic heterogeneity, it has been a major challenge to comprehensively investigate genetic causes of ID. The fruit fly *Drosophila* emerges as a very powerful organism for such endeavors. In this review, we aim to provide an overview on the contribution that *Drosophila* research has made to our understanding of ID, and thus to the genetic control of cognition. We further discuss future challenges and current limitations in ID-related research, including identification of molecular pathways that are disrupted in ID, bottlenecks in diagnostics, and development of therapeutic intervention. We specify how *Drosophila* can help to provide conceptually novel insights into these matters. Finally, we debate how the lessons learnt from ID research in *Drosophila* can be applied to other cognitive disorders with complex and poorly understood genetics.

2. Advantages and applications of *Drosophila* as a model for cognitive disorders

Understanding the function of the human brain is one of the main challenges in neuroscience. Researchers have turned toward organisms with more simple and easily manipulable brains to study fundamental brain processes on a molecular, cellular and circuit level. The *Drosophila* brain is, with approximately 100,000 neurons, relatively small yet sufficiently complex to provide a suitable model (Bellen et al., 2010). Despite the evolutionary distance between flies and humans, a strong conservation of genes, pathways and regulatory molecular networks has been demonstrated. 75% of human disease genes have related sequences in *Drosophila* (Bier, 2005). Correspondingly, we found unambiguous counterparts for 73% of ID genes in *Drosophila* (Oortveld et al., 2013).

In contrast to mammalian models, the generation of fly mutants is easy, cheap and fast. Moreover, the pool of publically available stocks that can be readily utilized to induce gain- and loss-of-function is enormous and steadily increasing (Bellen et al., 2010; Dietzl et al., 2007; Matthews et al., 2005). *Drosophila* is well suited for ID research, as it provides numerous approaches to investigate defects in neuronal morphology and function along with the possibility to assay cognitive processes. Below and in Fig. 1, we summarize how *Drosophila* can be used as a model to better understand the mechanisms underlying ID and other genetic disorders.

1. Gene function can be analyzed at a relatively high throughput by gene manipulation, followed by characterization of the resulting molecular, cellular and/or behavioral phenotypes. *Drosophila* mutant, knockdown, or overexpression strains can be ordered from public stock centers, or new strains can be generated.
2. The primary origin of a phenotype can be determined by cell type-specific gene manipulation, e.g. through the use of the UAS-GAL4 system (Brand and Perrimon, 1993; Duffy, 2002). Large collections of promoter elements and tools are available that can be used to individually manipulate specific tissues, subsets of cell populations, and even single cells.
3. Temporal requirements for gene function can be determined through manipulation with molecular switches that can flexibly be turned on or off. Such temporal information can be of crucial importance for the prospects of therapeutic intervention.

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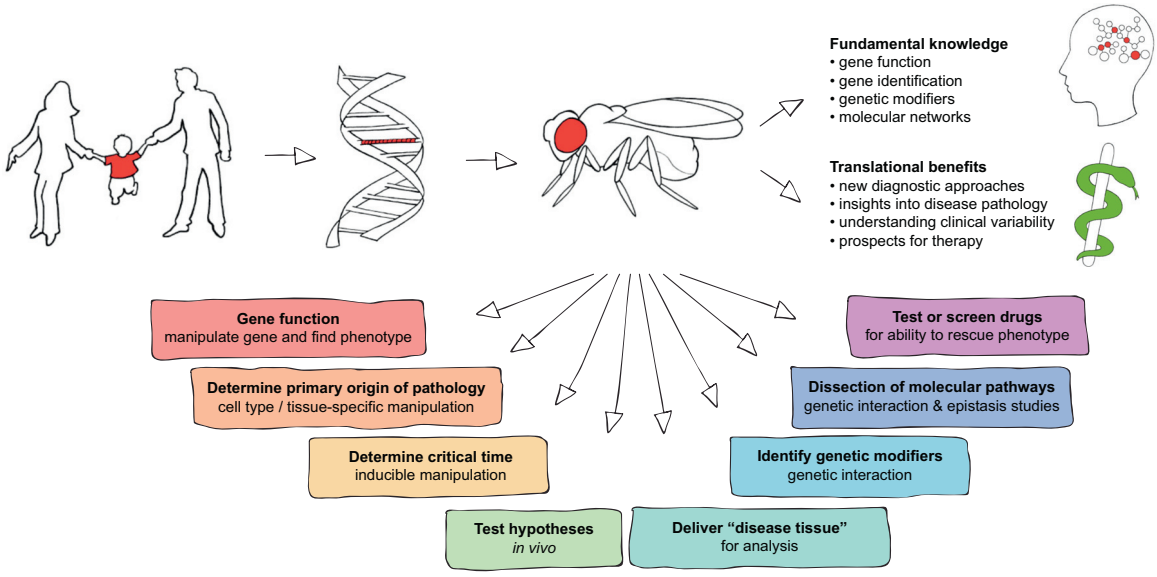


Fig. 1. Advantages and application of *Drosophila* as a disease model. The numerous approaches to investigate defects in neuronal morphology and function make *Drosophila* well suited to increase fundamental knowledge and provide translational benefits for ID.

4. Hypotheses can be tested in context of an intact organism. Such hypotheses can include everything from the presumptive regulation of one gene by another (e.g. using a molecular readout) to the regulation of a specific behavior by a certain type of neuron or neuronal circuit.
5. Disease-relevant tissues can be acquired for analyses in unlimited quantities. In brain research this is an important added value, since the human brain can only be studied post-mortem or with non-invasive imaging technology. The *Drosophila* brain or neuronal subtypes can be studied by various molecular and imaging techniques, down to subcellular resolution.
6. Genetic modifier screens can be used to isolate genes that genetically interact with the disease gene of interest. Such modifiers of fly disease model phenotypes represent candidate genes or risk factors for the same or related disease.
7. The possibility to genetically combine two or more mutations in different genes has made it possible to dissect molecular networks, for example the hierarchy of signaling pathways.
8. Finally, it is an excellent tool for testing large numbers of chemical compounds for their ability to alleviate fly phenotypes, paving the way for therapeutic trials in mammalian models and humans.

The fundamental knowledge that can be generated in this manner has great potential to find its way into the clinic (Fig. 1). Taken together, these features make *Drosophila* an extremely valuable organism to investigate human disease of the brain including ID.

3. *Drosophila* models of ID

At the beginning of the new millennium, *Drosophila* was used for the first time to address ID gene function. Wan *et al.* studied *Fmr1*, the *Drosophila* ortholog of the Fragile X mental retardation gene (Wan et al., 2000). *Drosophila* has now been used for over a decade to model ID, which has provided a large amount of disease-relevant information. In this chapter, we summarize some of these insights into ID gene function and pathology.

3.1. The contribution of behavioral tests to study ID

One of the advantages of working with an animal model such as *Drosophila* is that the effect of gene manipulation can be studied at the level of the whole organism. ID genes have been tested for behavioral defects, starting from relatively simple and proceeding to more complex behaviors. The role of some ID genes has been further dissected and mapped to specific tissues or neuronal circuits. A range of assays has been used to address simple fly behavior, including tracking of larval crawling and motor development, negative geotaxis, phototaxis, and flight assays. These assays are robust, relatively simple and quick to perform. Mutant flies can be tested for an effect on their general fitness and, when combined with cell-specific manipulation tools, for the requirement of a gene in a tissue/cell-type of interest. For example, in a flight assay *Fmr1* mutant flies display uncoordinated behavior, which has been compared to delayed motor development that is seen in some patients with Fragile X syndrome (Zhang et al., 2001). The *Drosophila* model of Angelman syndrome, a *Ube3a* mutant strain, has poor climbing ability indicative of motor dysfunction, resembling severe loss of motor coordination in patients with Angelman syndrome (Wu et al., 2008). However, the degree and pattern of neuronal dysfunction can differ between humans and *Drosophila*. For example, inactivating mutations in *Drosophila Nab2*, the ortholog of the human gene *ZC3H14*, cause defects in locomotion, whereas the patients are reported to have a non-syndromic ID phenotype (Pak et al., 2011).

Drosophila also offers plenty of opportunities to test more complex and cognitive behaviors, most importantly learning and memory. Since ID patients suffer from several aspects of impaired learning and memory capabilities (Bolduc and Tully, 2009), it is of great interest to investigate the role of ID gene orthologs in these processes. Indeed, the number of such studies has been increasing rapidly within the past years. The most commonly used paradigms were olfactory learning and courtship conditioning (Bolduc and Tully, 2009), but also spatial learning and habituation paradigms have been applied to ID models (see below). Learning and/or memory defects have been found associated with mutations in *Drosophila* orthologs of *GNAS* (Connolly et al., 1996), *NF1* (Guo et al., 2000), *FLNA* (Dubnau et al., 2003), *RSK2* (Putz et al., 2004), *FMR1* (McBride et al., 2005), *PRSS12* (Didelot et al., 2006), *UBE3A* (Wu et al., 2008), *PQBP1* (Tamura et al., 2010), *EHMT1* (Kramer et al., 2011), *NSUN2* (Abbasi-Moheb et al., 2012), *ANK3* (Iqbal et al., 2013), *CEP89* (van Bon et al., 2013) and *GATAD2B* (Willemsen et al., 2013).

Most studies so far have only utilized a single paradigm to investigate learning and/or memory in a specific ID model, whereby the choice of the assay was not based on the clinical phenotype or

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biology of the gene but rather determined by the expertise of the investigating laboratory. It is interesting to note that some multi-paradigm studies found distinct mutant phenotypes, depending on the type of mutation and behavioral paradigm tested. For example, *Drosophila S6kII* mutants were tested in two learning tasks: operant place learning and Pavlovian olfactory conditioning (Putz et al., 2004). The S6kII protein is part of the Ras extracellular signal-regulated kinase (ERK) signaling pathway and the human ortholog *RPS6KA3* is associated with Coffin-Lowry syndrome. In three tested *S6kII* mutants, expression was reduced through a P-element insertion, a partial, or a full gene deletion. The P-element insertion mutant only affected operant place learning. The partial deletion mutant, lacking part of the N-terminal kinase domain, performed poorly in both learning tasks. The null mutant surprisingly only affected olfactory learning. These findings suggest that the type of allele can determine the behavioral read-out, even when all alleles presumably represent (partial) loss-of-function conditions. This appears analogous to the human situation where extensive clinical variability is associated with allelic mutations. Work on *EHMT* mutant flies, the *Drosophila* model of Kleefstra syndrome, illustrates that the function of a gene also depends on the behavioral context. *EHMT* mutants show defects in habituation, a form of non-associative learning, whereas learning in the courtship conditioning paradigm is unaffected (Kramer et al., 2011). Differences between paradigms have also been found in classic learning and memory mutants (for a review see e.g. (Engel and Wu, 2009)) and may depend on expression patterns of the gene, architecture of the behavior-underlying circuit or different neuronal properties. This makes it difficult to impossible, with current knowledge, to rationalize which paradigm to preferably use when modeling ID.

The combination of cognitive tests and genetic approaches in *Drosophila* can be used to distinguish between genes that execute a specific neuronal function in cognitive processes and genes that act elsewhere, causing ID in an indirect manner. This is of particular interest when ID patients present with a broader spectrum of clinical features, like Kleefstra syndrome where ID patients present with developmental delay, hypotonia, distinct facial and other features (Willemsen et al., 2012). Transgenic re-expression of *EHMT* in all neurons ('pan-neuronally') rescued courtship memory defects in *EHMT* mutant flies, indicating a direct role of *EHMT* in cognitive processes (Kramer et al., 2011). Very similarly, the autosomal-recessive ID gene *Nsun2*, associated with microcephaly and additional clinical features, was shown to be essential for short-term memory in the aversive olfactory conditioning paradigm. The phenotype was rescued by pan-neuronal re-expression of *Nsun2* in the mutant background (Abbasi-Moheb et al., 2012).

The same and other conditional strategies, often exploiting the UAS-GAL4 system, can be applied to identify the subset of neurons that are important for specific behaviors (Fig. 1). A brain structure called the mushroom body is known to be required for several forms of learning and memory in *Drosophila*. Re-expression of *EHMT* in mushroom body neurons was sufficient to rescue courtship memory defects shown by *EHMT* mutants (Kramer et al., 2011). Mutant flies of the neurotrypsin-related gene *Tequila* display defective long-term memory in the olfactory learning paradigm (Didelot et al., 2006). RNAi-mediated knockdown of *Tequila* in mushroom body neurons was sufficient to cause the defects. Likewise, *Wah*, the ortholog of *KANSL1*, and *simj*, the ortholog of *GATAD2B*, were investigated. Mushroom body-specific knockdown of *wah* reduces learning ability in courtship conditioning and pan-neuronal knockdown of *simj* decreases light-off jump reflex habituation (non-associative learning) (Koolen et al., 2012; Willemsen et al., 2013). In addition, 10 out of 37 *Drosophila* orthologs of recently identified ID candidate genes (Najmabadi et al., 2011) are necessary for normal long-term memory in courtship conditioning, as found by pan-neuronal RNAi experiments (K. Keleman, personal communication).

In summary, learning and memory function is affected in a significant number of ID models. Such findings and models provide additional evidence for the role of the investigated genes in cognitive processes, and allow for further dissection of the underlying molecular and cellular defects.

3.2. Neuronal development and architecture

Drosophila offers many opportunities to study the role of genes in the organization of the brain and its nervous system in greater detail. Here we provide an overview on how structural information on synapses, dendrites and axons helps to further understand the cellular basis of ID.

A significant number of ID genes encode proteins involved in synapse biology (van Bokhoven, 2011). Because of this observation and since synaptic morphology and function is important for cognitive processes, many studies put emphasis on determining the role of ID genes at a *Drosophila* synapse. The *Drosophila* neuromuscular junction (NMJ) has been widely used to address questions related to mechanisms underlying neurotransmitter release, synapse formation and physiology (Ruiz-Canada and Budnik, 2006a). Like many central excitatory mammalian synapses, the *Drosophila* NMJ is glutamatergic, contains similar molecular components and is regulated by orthologous pathways. The synaptic terminals of the NMJ are organized in boutons and each bouton contains numerous presynaptic release sites, known as active zones. Zhang et al. showed that loss of FMR1 causes synaptic overgrowth, whereas overexpression causes undergrowth, with fewer (but larger) synaptic boutons (Zhang et al., 2001). Based on phenotypic similarities, a functional link between Fmr1 and futsch, a microtubule-associated protein, was hypothesized and confirmed by a double mutant condition that reversed the *Fmr1* mutant overgrowth phenotype. The importance of the microtubule network in NMJ development and function was further underscored by a study that investigated the tubulin-specific chaperone E gene (*TBCE*), which is linked to the congenital hypoparathyroidism-retardation-dysmorphism (HRD) syndrome (Jin et al., 2009). *Drosophila tbce* was found to function both pre- and postsynaptically. Knockdown at either side of the NMJ caused an increase in the number of branches and boutons, whereas the bouton area was smaller. This observation corresponds with the hypothesis that synaptic growth depends on microtubule dynamics, but not necessarily on the absolute quantity of microtubules (Ruiz-Canada and Budnik, 2006b). In addition to microtubule-related ID genes, NMJ structural abnormalities have recently been described in ID fly models of mitochondria-related proteins, such as Cep89 and its *Drosophila* ortholog CG8214 (van Bon et al., 2013), UbcD6 and its *Drosophila* ortholog Rad6a (Haddad et al., 2013), adhesion molecules like the Neurexins (Zweier et al., 2009), intracellular phospholipases (Schuurs-Hoeijmakers et al., 2012) and proteins involved in chromatin remodeling, like KANSL1 and GATAD2B and their *Drosophila* orthologs wah (Koolen et al., 2012) and simj (Willemsen et al., 2013), respectively.

Beyond morphological analyses, the NMJ is a suitable model to directly measure the effect of mutations on neurotransmission. Larval muscles and motor nerves are relatively large; this genetically traceable synapse is therefore fully amenable to electrophysiological analyses. For example, the combination of pre- versus postsynaptic manipulation and electrophysiology revealed that tbce is exclusively required presynaptically for normal neurotransmission. Altered levels of presynaptic tbce results in an increase in the excitatory junction potential (EJP) and miniature EJP (mEJP) amplitudes, whereas postsynaptic manipulation does not change these parameters (Jin et al., 2009). In a large-scale screen focused on postsynaptic glutamate receptor localization,

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mutations in *rt*, the *Drosophila* ortholog of *POMT1*, were found to cause a reduced level of GluRIIB (Wairkar et al., 2008). Mutations in *POMT1* are linked to muscular dystrophy-dystroglycanopathies with ID (van Reeuwijk et al., 2006). *Drosophila rt* synapses show decreased synaptic strength and release probability but no structural NMJ defects. These defects were only rescued when rt was simultaneously re-expressed pre- and postsynaptically (Wairkar et al., 2008). Moreover, it was demonstrated that rt is required to glycosylate the *Drosophila* dystroglycan ortholog Dg in vivo, which likely is essential for proper synapse function.

The giant fiber system (GFS) has been used to study morphology and function of central synapses. The GFS mediates the escape response of adult flies and connects with the dorsal longitudinal motoneurons and the Tergo Trochanteral Muscle neuron (TTMn). This system was used to study the role of *Drosophila Nrg*, whose ortholog *L1CAM* is associated to a variety of neurological diseases, in central synapse formation (Godenschwege et al., 2006). Whereas *Nrg* null mutants displayed severe axon guidance defects that precluded synaptic analyses, flies with the homozygous missense mutation Nrg^{849}, located at a similar position as one of the many identified human pathological mutations, showed such defects only with low penetrance. The Nrg^{849} mutants were characterized by structurally and functionally compromised synaptic connections between GFS and TTMn neurons (Godenschwege et al., 2006; Hortsch et al., 2009). Expression of vertebrate L1CAM simultaneously at both the pre- and postsynaptic site was able to rescue these synaptic defects (Godenschwege et al., 2006).

The *Drosophila* central synapse of the VS1 neuron of the lobula plate vertical system was recently found to be regulated by the Fragile X protein Fmr1. The VS1 neuron shows small actin-enriched protrusions that are morphologically and functionally similar to dendritic spines, mammalian postsynaptic compartments, making this synapse an attractive postsynaptic system to study in ID models. In wild-type flies, VS1 spine number and length increase after sleep deprivation and show pruning when flies are allowed to sleep. FMR1 is required for this sleep-dependent synaptic renormalization. This study provided compelling structural evidence for sleep regulating synaptic homeostasis and for involvement of an ID gene therein (Bushey et al., 2011).

Changes in dendritic arborization are commonly observed in ID patients (Kaufmann and Moser, 2000) and might contribute to their cognitive impairment. The dendritic arborization (DA) neurons in *Drosophila*, also named multiple dendritic neurons, are a well-suited model to study dendrite architecture and arborization. DA neurons, particularly the morphologically complex class IV DA neurons (Jan and Jan, 2010), have been examined in a handful of fly models of ID genes. *Fmr1* mutants show an elevated number of higher-order dendritic branches, reintroducing one copy of the wild-type *Fmr1* gene reversed the phenotype. Overexpression of *Fmr1* has the opposite effect, revealing a dosage-dependent mechanism (Lee et al., 2003). Loss of *Ube3a* or *EHMT* in the Angelman and Kleefstra syndrome fly models results in reduced dendritic branching, which suggests that a dendritic pathology might contribute to the human phenotypes (Kramer and van Bokhoven, 2009; Lu et al., 2009). More recently dendrite defects were also reported in the *Ube3a* and *Ehmt1* mouse models (Balemans et al., 2013; Miao et al., 2013). The importance of dendrite morphology in the etiology of ID was also applied to strengthen the hypothesis that haploinsufficiency of the *CDK19* gene causes ID with microcephaly and congenital retinal folds in a single patient. Knockdown of the *CDK19 Drosophila* ortholog *Cdk8* in DA neurons causes reduced dendritic fields and reduced dendritic complexity, supporting the link of *CDK19* to the ID phenotype (Mukhopadhyay et al., 2010).

Outgrowing axons follow a precise path during development to connect to other neurons via their terminal synapses. Connections must be made with the correct targets, which is challenging considering the distance and large number of pathfinding choices. It is likely that axonal biology, like other aspects of neuronal morphology, plays an important role in the etiology of ID. White matter tracts in the human brain, rich in myelinated axons, can be visualized using diffusion tensor imaging (Huang and Vasung, 2013). However, due to technical limitations, including lack of a method analogous to dendritic Golgi staining that would allow tracing of single axons, this field in humans remains largely unexplored. *Drosophila* can be used to study the processes of axon growth, pathfinding and branching by labeling subsets or single axons. Work on the *Drosophila* Fragile X model played a pioneering role linking *Fmr1* to these processes. Loss of *Fmr1* strongly affects neurite extension and axon guidance of both dorsal cluster and lateral neurons, but not photoreceptor neurons (Morales et al., 2002). Ectopic axon branching patterns and axon overgrowth have also been observed in neurons of the mushroom body (Michel et al., 2004; Pan et al., 2004). In mutant *Fmr1* mushroom body γ neurons, these phenotypes have been shown to result from defects in activity-dependent axon pruning (Tessier and Broadie, 2008). Evidence for axonal functions of the Fragile X protein also accumulates in mammalian systems (Section 4.2). It is to be hoped that further progress in human brain imaging techniques in combination with functional data coming from model organisms will shed more light on the contribution of axonal biology on ID.

3.3. ID gene function and circadian rhythm

Apart from defects in the above-discussed aspects of neuronal development and function that are common to at least some ID models, work in *Drosophila* linked ID gene function to circadian rhythm. *Fmr1* mutant flies are arrhythmic, with an erratic pattern of locomotor activity including periods of hyperactivity, and defects in circadian period upon overexpression (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002). Fmr1 was shown to be required for normal cAMP response element-binding protein (CREB) activity, downstream of clock neurons (Dockendorff et al., 2002). Subsequently, circadian rhythm defects were also found for the mammalian Fragile X protein family (in *Fmr1*/*Fxr2* double mutant mice, hetero- and homozygotes) (Zhang et al., 2008). Alterations in the circadian rhythm are also increasingly considered as a cause rather than a consequence of neuropsychiatric disorders (Menet and Rosbash, 2011). Given the observed sleep disorders in Fragile X patients and the overlap of ID with neuropsychiatric disorders (further discussed in chapter 7) alterations in circadian rhythms might contribute to behavioral alterations in ID patients (Zhang et al., 2008). Circadian rhythm defects in other ID models such as *Ube3a* mutant flies (Wu et al., 2008) further support this idea.

3.4. Defective RNAi machinery and ID

Drosophila work on Fragile X syndrome pioneered the link between the RNAi machinery and human disease. Two back-to-back papers published in 2002 discovered an association between Fmr1 and the RNA-induced silencing complex (RISC), and the RNase III enzyme Dicer, implicating Fmr1 as part of the RNAi-related apparatus (Caudy et al., 2002; Ishizuka et al., 2002). Similarly, Dicer and eIF2C2, the mammalian ortholog of Argonaute-1 (*AGO1*), were also found to interact with mammalian FMRP (Jin et al., 2004). The identification of biochemical and genetic interactions of the Fragile X protein with components of the miRNA pathway, the

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latter demonstrated in *Drosophila*, could point to a mechanism through which the protein exerts its function (Jin et al., 2004).

3.5. Contributions of fundamental biological studies

Many studies of *Drosophila* ID gene orthologs have been performed without the explicit intent to investigate their associated disease. Examples of 'incidental ID models' include studies where disease gene orthologs were found in unbiased screens to cause neuronal phenotypes. Such screens have provided unexpected insights into ID, such as the discovery of a post-mitotic role for subunits of the cohesin complex, well known for their role in sister-chromatic cohesion during meiosis and mitosis (Schuldiner et al., 2008). The cohesin complex subunits *NIPBL*, *SMC1A*, *SMC3* and *RAD21* are mutated in Cornelia de Lange syndrome (CdLS). *Drosophila* studies revealed a function in axon pruning in the mushroom body and dendrite targeting in olfactory projection neurons. This post-mitotic role of cohesin was found to be mediated through altered gene expression (Pauli et al., 2008; Schuldiner et al., 2008). In addition, a mosaic screen in the *Drosophila* wing epithelium has uncovered a role for *SMC3* in planar cell polarity (PCP) an important process in development and function of the nervous system (Mouri et al., 2012; Tissir and Goffinet, 2013).

Incidental ID models also arise when genes have been investigated in *Drosophila* prior to disease implication. The *Drosophila* minibrain (mnb) kinase was reported in 1984 to have a critical role in *Drosophila* brain development. *mnb* mutants have a reduced brain volume of 40–50% compared to controls, with a drastic reduction in cell numbers (Fischbach and Heisenberg, 1984). It was not until 24 years later that de novo truncations in the human *mnb* ortholog *DYRK1A* were identified in unrelated patients with a clinical phenotype including a significantly reduced brain size (microcephaly) (Møller et al., 2008). Another example is the class I helix-loop-helix transcription factor daughterless (da) that controls expression of important neuronal genes and is required for neural differentiation in *Drosophila*. The association to disease came to light 19 years later when mutations in the human ortholog *TCF4* were found to cause Pitt-Hopkins syndrome (PTHS), a severe ID disorder (Amiel et al., 2007; Brockschmidt et al., 2007; Caudy et al., 1988; Zweier et al., 2009). *Drosophila Ankyrin-2*, the fly ortholog of human *ANK2* and *ANK3*, was found to be required for synapse stability in genetic screens. Mutants show synaptic disassembly and retraction and disrupted neuronal excitability (Koch et al., 2008; Pielage et al., 2008). Recently, mutations in *ANK3* were identified in patients with ID and the cognitive defects in humans were supported by memory defects in *Drosophila* (Iqbal et al., 2013). These findings highlight the importance and direct relevance of fundamental studies in *Drosophila* to human disease.

3.6. Concluding remarks

The Fragile X mental retardation gene was the first ID gene to be studied in *Drosophila*, and to date still is the most frequently studied (Fig. 2). Phenotypes that have been found in the Fragile X fly have guided research on other *Drosophila* ID models, such as fly models of the X-linked Alpha thalassemia/mental retardation syndrome (*ATRX*) and genes involved in dystroglycanopathies (Fig. 2) (Fradkin et al., 2008; Lee et al., 2007; Marrone et al., 2011; Wairkar et al., 2008; Zhan et al., 2010) and many others as discussed above. This chapter provided an overview on how *Drosophila* has been used as a model to study ID. How *Drosophila* can be exploited as a model for ID in future fundamental and translational research is discussed below.

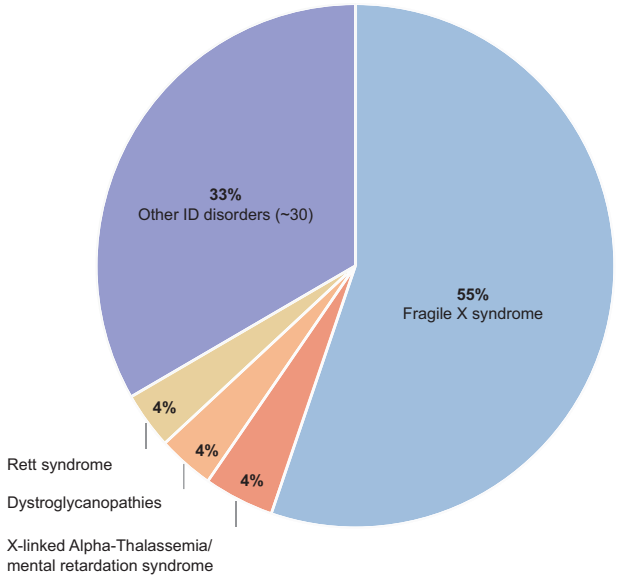


Fig. 2. Thirteen years of publications for *Drosophila* models of intellectual disability. Thirteen years of *Drosophila* as a model to study intellectual disability (2000–June 2013) yielded 114 publications, from which more than half ($n = 63$) describe Fragile X syndrome, the most extensively studied ID disorder. Other frequently investigated syndromes are X-linked alpha-thalassemia/mental retardation syndrome ($n = 5$), Dystroglycanopathies ($n = 4$) and Rett syndrome ($n = 4$). The around 30 other ID disorders are represented by less than four publications per disorder.

4. Molecular pathways and functional modules in cognitive (dys)function

4.1. ID genes – different pieces of the same puzzle

Perhaps one of the most important and exciting outcomes of ID research is the accumulating evidence that ID genes encode proteins that operate together in common complexes, pathways or networks. Diverse cellular functions and mechanisms have emerged, including intracellular signal transduction pathways that regulate presynaptic pathways, postsynaptic protein complexes, cytoskeleton dynamics, epigenetic modulation of chromatin structure, and transcription (van Bokhoven, 2011).

The pathways most prominently affected by proteins implicated in ID are Rho and Ras small GTPase signaling cascades. Rho GTPases are key regulators of actin remodeling. The regulation of the actin cytoskeleton is important for many aspects of neuronal morphology and function, including remodeling and plasticity of actin-rich dendritic spines. Dendritic spine plasticity is known to correlate with learning and memory, and abnormal dendritic spines are a common feature of ID (Kaufmann and Moser, 2000; Lamprecht and LeDoux, 2004). ID genes/proteins operating in Rho GTPase pathways belong to the classes of Rho GTPase activating proteins (GAPs: *PHN1*, *SRGAP3*/*MEGAP*, *OCRL1*), Rho guanine nucleotide exchange factors (GEFs: *ARHGEF6*, *ARHGEF9*, and *FGD1*) and Rho GTPase effector proteins (*PAK3*) (Dierssen and Ramakers, 2006; Nadif Kasri and Van Aelst, 2008; Schenck et al., 2004).

The Ras/MAPK pathway is part of cellular signaling cascades and has pleiotropic functions at the pre- and postsynaptic site. Presynaptically Ras/MAPK regulates neurotransmitter release at the axon terminal and depending on the type of neuron, excitatory or inhibitory, a similar mutation can have opposite downstream effects. Postsynaptically the Ras/MAPK pathway functions as a signal integrator at the dendritic spines, where it regulates transcription and translation events (Thomas and Huganir, 2004). The transcription regulation through transcription factor CREB is essential for many forms of learning and memory (Lonze and Ginty,

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2002). Genes that are mutated in ID are the Ras GTPases (*HRAS* and *KRAS*), downstream effectors of Ras (*RAF1*, *BRAF*, *MEK1*, *MEK2*, and *RPS6KA3*/*RSK2*) and regulators of the Ras/MAPK pathway (*SHP2*, *SOS1*, *NF1*, *SPRED1*, *SHOC*). The clinically overlapping disorders are commonly referred to as Rasopathies (Krab et al., 2008b).

Additional pathways in ID have emerged. Among the more than 20 ID genes that encode chromatin remodeling factors are several that physically interact and/or functionally cooperate (van Bokhoven, 2011). MECP2 for example, the methyl-CpG-binding protein mutated in Rett syndrome, has been found in complexes with ATRX/XNP, DNMT3B, CREBBP, RPS6KA1/RSK, and CDKL5 (Kramer and van Bokhoven, 2009).

The examples listed above show that identifying these pathways greatly improves our knowledge of involved mechanisms, contributing to our understanding of the patient phenotype. However, the so far emerging pathways are among the best-investigated cellular signaling pathways. It will be more challenging to unravel the role of less investigated or unknown pathways underlying ID. This will be further discussed in the next section.

4.2. *Drosophila* to define molecular pathways and networks underlying ID

One of the most successful applications of classic *Drosophila* genetics is modifier screens. The aim of modifier screens is to identify second site mutations that modify a phenotype caused by a gene of interest. Such unbiased screens can reveal functionally related genes, and thereby additional components of a pathway. A seminal example is the above-mentioned Ras/MAPK pathway, which was described and dissected in the context of *Drosophila* eye development (Karim et al., 1996; Therrien et al., 2000). The same principle can also be utilized in a targeted manner to test for genetic interactions between potentially connected genes.

Loss of the Fragile X protein causes altered synapse morphology in human patients, mice and *Drosophila* models (Zarnescu et al., 2005). Research in *Drosophila* revealed that the Fragile X protein, via the Rac1 effector protein CYFIP/Sra-1, is linked to actin-remodeling Rho GTPase pathways (Schenck et al., 2003). It was shown that CYFIP/Sra-1 acts as a Rac1 effector that antagonizes Fmr1 function at the *Drosophila* NMJ. This study provided the first link between signal-dependent cytoskeleton remodeling and Fragile X syndrome, raising the interesting possibility that actin remodeling and local protein synthesis at synapses, two crucial processes in synaptic plasticity, are directly co-regulated (Schenck et al., 2003). Further connections between the Fragile X protein and core players of actin remodeling pathways have been subsequently revealed. Expression of *Drosophila* Rac1 and profilin were found to be controlled by Fmr1, and further findings in mammalian systems placed additional emphasis on defects in actin misregulation as a major problem in Fragile X syndrome and its neuropathology (Castets et al., 2005; Chen et al., 2010; Lee et al., 2003; Reeve et al., 2005)

Another link was recently described between the Fragile X protein and the Down syndrome cell-adhesion molecule (Dscam). DSCAM is upregulated in Down syndrome and believed to contribute to the neuronal pathology of Trisomy 21 (Alves-Sampaio et al., 2010; Rachidi and Lopes, 2007). The protein is involved in intraneuronal self-recognition, axon guidance and synaptic target selection (Hattori et al., 2008). *Drosophila* was used as a model to study the mechanisms via which Dscam exerts these conserved functions. Following up on earlier findings that identified Dscam as an RNA target of Fmr1, the authors found that Fmr1 suppresses Dscam translation to restrict presynaptic arbor growth in a dose-dependent manner (Brown et al., 2001; Darnell et al., 2011; Kim et al., 2013a). This is in agreement with a second study that reported that increased Dscam protein levels, either by Dscam overexpression or by reduced Fmr1 levels, results in axonal targeting errors in pSc mechanosensory neurons (Cvetkovska et al., 2013). Moreover, reducing Dscam levels in Fmr1 null flies reduces synaptic targeting errors and rescues behavioral defects. Excess Dscam protein may thus be a common molecular mechanism that underlies altered neural wiring in both disorders (Cvetkovska et al., 2013). These studies added to an increasing body of evidence that Fmr1, in addition to its dendritic and postsynaptic functions, plays important roles presynaptically and in axons (Contractor, 2013; Patel et al., 2013).

Shared ID-associated clinical phenotypes, if sufficiently characteristic, can generate important novel hypotheses with respect to proteins that functionally cooperate. Such hypotheses are testable in *Drosophila*. To date, mutations in three genes have been shown to cause Pitt-Hopkins syndrome (PTHS) and phenotypically overlapping disorders. These genes encode the transcription factor TCF4, the presynaptic adhesion protein NRXN1, and the distal neurexin-family member CNTNAP2 (Zweier et al., 2009, 2007). Whereas the involvement of *NRXN1* suggests that PTHS is a synaptopathy, *CNTNAP2* organizes nodal microdomains of myelinated axons and evidence for a synaptic role of the encoded protein has been lacking. Using *Drosophila* as a model, it was shown that overexpression of either protein, Neurexin-1 ortholog (Nrx-1) or CNTNAP2 ortholog (Nrx-IV), reorganizes synaptic (NMJ) morphology and induces increased density of active zones, the synaptic domains of neurotransmitter release. Moreover, dosage of either protein determines the level of the presynaptic active zone protein bruchpilot, indicating a possible common molecular mechanism in Nrx-1 and Nrx-IV mutant conditions. These findings and the observation of anti-Nrx-IV immunoreactivity at the investigated synapse argue that a shared synaptic mechanism contributes to the similar clinical phenotypes in *NRXN1* and *CNTNAP2* patients (Zweier et al., 2009). Novel roles for *CNTNAP2*, including a function in synapse organization, have subsequently also been reported in mammalian neurons (Anderson et al., 2012). Of note, the phenotypic commonalities caused by mutations in *NRXN1* and *CNTNAP2* extends beyond PTHS. Milder conditions (heterozygous variants or copy-number variations) have been implicated in an overlapping multitude of neuropsychiatric disorders, including Autism Spectrum Disorder (ASD) and Schizophrenia (Zweier et al., 2009). This also matches the role of *TCF4*, which in a recent cross-disorder analysis by the Psychiatric Genomics Consortium, has also been associated to ASD and Schizophrenia (Psychiatric Genomics Consortium et al., 2013). Interestingly, it has been recently shown that TCF4 can transactivate the *NRXN1β* and *CNTNAP2* promoters, suggesting that these genes are under direct control of TCF4 (Forrest et al., 2012). The molecular mechanisms that link the three PTHS genes and the question in how far defects in neuronal differentiation versus post-mitotic neuronal function contribute to ID in PTHS patients warrants further investigation. The latter has important consequences for therapeutic intervention strategies (see also chapter 6).

Drosophila has also been successfully applied to extend the above-mentioned MECP2 functional network (see section 4.1), even though MECP2 itself is not conserved in *Drosophila*. Genetic interaction experiments in transgenic flies expressing human MECP2 reveal that reduced dosage of *osa* is capable of suppressing dendritic defects caused by altered MECP2 levels (Vonhoff et al., 2012). *Osa* is the common *Drosophila* ancestor of human *ARID1A* and *ARID1B* genes, subunits of the SWI/SNF complex that have recently been shown to cause Coffin-Siris syndrome (Santen et al., 2012; Tsurusaki et al., 2012). Mutations in *ARID1B* have also been reported as a frequent cause of moderate to severe ID with additional clinical features and have been implicated in ASD (Hoyer et al., 2012; O'Roak et al., 2012). Among the other identified modifiers of MECP2 is *pbl*, a target of Ube3a, involved in Angelman syndrome as discussed above (Cukier et al., 2008). Rett and Angelman syndromes

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are both devastating neurological disorders that share, in addition to ID, a number of other clinical features, suggesting a possible mechanistic overlap. Indeed, reduced Ube3a levels are able to suppress a MECP2 gain-of-function eye phenotype, MECP2 and Ube3a proteins physically interact and regulate the expression of shared target genes (Kim et al., 2013b). Thus, *Drosophila* studies have established connections between MECP2 and other ID genes.

4.3. Addressing other signatures common to (subsets of) ID disorders

Identifying molecular mechanisms that are shared by ID disorders could provide highly valuable information in several aspects: (1) for our fundamental understanding of the biology underlying ID, (2) as biomarkers with potential applications in future diagnostics, and (3) to define common targets for future therapeutic intervention. Such common mechanisms do not necessarily need to represent signaling pathways in which ID gene products directly operate. Shared transcriptional or metabolic dysregulation and other molecular 'signatures' can be important as well, even if indirectly caused. First reported examples of such signatures include altered immediate early gene expression through mutated ID genes *MED23*, *MED12* or *ERCC2*/*XPD* (Hashimoto et al., 2011), and decreased tryptophan levels in ASD patients with and without ID (Boccuto et al., 2013).

It can be expected that future studies will report other defects that may commonly occur in subsets of ID and/or other cognitive disorders. *Drosophila* models make a suitable and rather unlimited "test cohort" to comprehensively replicate and validate such findings, e.g. through biochemical approaches and metabolic measurements in a large number of ID models. *Drosophila* experiments can also provide direct evidence for the contribution of identified signatures to a relevant phenotype.

4.4. Large-scale studies to identify novel ID networks and their function

Drosophila is well suited to generate large comparative phenotypic datasets, because of the relative ease in which forward and reverse genetic screens can be performed. In vivo screening provides additional benefits over cell-based models, because complex phenotypes such as cognition can be assayed.

Screens can be used to identify missing components in specific processes or pathways, but they can also be used to obtain a broad overview on function of a defined group of insufficiently characterized genes. Performing large-scale reverse genetic screens, in combination with extensive phenotyping, could provide an overview of the role of ID genes within the nervous system. In addition, performing large-scale phenotyping analyses in model organisms, compared to human patients, has several benefits. First, many of the conditions are rare, thus the small amount of patients that can be investigated is limiting. Second, patients are usually seen by different clinicians across various countries, which might result in inconsistent clinical analysis between patients. Third, one cannot control for genetic background and environmental factors in patients, whereas in model organisms such as *Drosophila* the genetic background can be selected, and flies can be raised under controlled conditions. Combined, this keeps the influence of variants other than the gene in question to a minimum. Thus, comparative phenotyping approaches in model organisms should be able to significantly contribute to the identification of evolutionarily conserved genotype-phenotype correlations that are relevant to human disorders.

Based on the widely recognized principle that identical or similar phenotypes arise from disruptions of common processes, comparative phenotyping approaches should be able to predict novel gene functions and map previously unappreciated functional modules and pathways. A recent study reported an RNAi-based candidate screen, targeting genes involved in a range of neurological disorders, for defects in synaptic and mitochondrial function. Loss of *UbcD6*, the *Drosophila* ortholog of ID gene *UBE2A*, induces a number of phenotypes that, unexpectedly for its established role in the nucleus, resembles defects found in Parkin mutants (Haddad et al., 2013). This phenotypic resemblance is due to an overlap in function: *Drosophila* UbcD6 has been demonstrated to act as an E2 ubiquitin-conjugating enzyme that, in combination with an E3 ubiquitin ligase such as Parkin, ubiquitinates mitochondrial proteins to facilitate the clearance of dysfunctional mitochondria, preventing neuronal dysfunction (Haddad et al., 2013). A second study systematically targeted 270 ID gene orthologs in the *Drosophila* eye (Oortveld et al., 2013). Assessment of neuronal function in behavioral and electrophysiological assays and multiparametric morphological analysis assigned many novel functions to ID genes, including a requirement in basal neurotransmission for 16 ID genes that had not previously been implicated in this process in any system or organism. Grouping genes by their phenotypes identified 26 highly connected functionally coherent modules that comprise a total of 100 ID genes and successfully predict additional gene functions such as a role in synapse/NMJ development. Interestingly, *Drosophila* phenotype groups show, in addition to ID, significant phenotypic similarity also in humans, indicating that the identified functional modules are conserved. This study provides a first overview of the modular architecture of ID and has presented unbiased evidence for a long suspected broad functional coherence underlying ID.

The indicated two studies pose the question whether RNAi is an appropriate tool to model ID disorders. Whereas loss- versus gain-of-function approaches in targeted studies should always be tailored to the clinical condition of interest, an inventory reported by Oortveld et al. revealed that for the vast majority of ID genes (partial) loss-of-function is thought to be the causative mechanism (Oortveld et al., 2013). This supports RNAi approaches as a suitable approximation to the large group of human ID disorders. Future systematic screens will address ID gene function in further disease-relevant contexts.

5. Clinical application of *Drosophila* I: gene identification and diagnostics

Drosophila has been used to identify genetic interactors of disease genes. Such research frequently aims to deliver candidate genes that can either modify the human disease phenotypes or directly cause (the same or a similar) disease. While this approach will remain valid, the need for candidate genes has become obsolete. With the advent of next-generation sequencing, human genetics generates more candidates than at present can be interpreted. Here, we anticipate an increasing contribution of *Drosophila* to the interpretation of genetic variants and their pathogenicity.

5.1. Challenges in gene identification in the era of next-generation sequencing

The advent of next-generation sequencing technology, which allows interrogation of unlimited amounts of genomic information with base-pair precise resolution, has begun to revolutionize human genetics. A number of ID syndromes were among the first diseases genetically solved by applying this technology after exome capture, including Schinzel-Giedion syndrome (SETBP1), Kabuki syndrome (*KMT2D*), and Baraitser-Winter syndrome (ACTB and ACTG1) (Hoischen et al., 2010; Ng et al., 2010a; Riviere et al., 2012). Common to these three and further successful studies is that

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sequencing has been performed in a carefully selected, phenotypically coherent cohort of patients. This strategy strongly increases the chances of finding the same gene to be affected in multiple patients, thereby validating its causative nature. However, this strategy cannot – or only to a minor extent – be applied to very rare, phenotypically heterogeneous or even non-syndromic ID cohorts. In many cases, suspicious variants are observed only in single patients or families (de Ligt et al., 2012; Najmabadi et al., 2011; Vissers et al., 2010). Furthermore, even considering only de novo mutations, multiple potential disease-causing variants can be identified within a single patient. The primary challenge in such studies has shifted from data analysis to interpretation, so as to distinguish between background variants and pathogenic mutations. Currently applied filters that identify potential causal variants among all variants detected are based on variant frequency, conservation scores, predicted deleteriousness to protein structure, and if available, evaluation of functional data of the affected gene (Ng et al., 2010b). Surveys carried out on copy-number variations have demonstrated, unsurprisingly for biologists, that functional information collected in model organisms is particularly powerful (Hehir-Kwa et al., 2010; Webber et al., 2009). The required functional data is available however for only a minority of genes, such as non-systematically acquired information on 5000 mouse genes. This limitation holds particularly true if only considering information relevant to the disease-relevant tissue(s), such as the nervous system for ID disorders. The paucity of relevant functional data has become a major bottleneck for the interpretation of variants in research and in routine diagnostics.

5.2. Guiding human genetics and clinical decision-making through functional studies in Drosophila

Next-generation sequencing technology has already reached diagnostics and in the following years is expected to become a standard in medical genomics (Gonzaga-Jauregui et al., 2012). The need for functional investigation of disease genes and variants can safely be assumed to explode, at least (but probably not only) in cases where human genetics/genomics will fail to detect recurrent changes.

Can animal models contribute to routine diagnostics? We believe so, but to overcome the bottleneck of lacking functional data, extraordinarily efficient model organisms are required that permit the generation of relevant information in a short time and on demand.

5.2.1. Support for causality through disease-relevant gene function

Drosophila is in an excellent position to lend ad hoc support to the causative nature of identified ID gene candidates that are identified in diagnostics. Phenotypes that were previously reported in fly models of well-established ID disorders, such as synaptic, learning and memory defects, have been tested in a number of *Drosophila* models of newly identified ID genes with various degrees of (human) genetic evidence.

An ID gene that has received support for causality by various *Drosophila* phenotypes including synaptic defects and learning deficits is *CEP89*, a gene homozygously deleted in a single patient with isolated complex IV deficiency, intellectual disability and multisystemic problems (van Bon et al., 2013). Other examples include the combined approaches of heterozygosity mapping and next-generation sequencing that led to the identification of *ANK3* and *NSUN2* as mutated in two forms of autosomal recessive ID. A balanced translocation that disrupts one copy of all *ANK3* isoforms in one individual, and a homozygous mutation in the longest *ANK3* isoform in a family were found. The causality of mutations in *ANK3* is supported by its previous implication in neuropsychiatric disorders in humans and by *Drosophila* studies revealing a role for this gene in synapse development (Koch et al., 2008; Pielage et al., 2008) and memory (Iqbal et al., 2013). One splice site and two missense mutations were found in the RNA methyltransferase *NSUN2* gene in patients with autosomal recessive moderate to severe ID and facial dysmorphism. The *Nsun2* fly model shows severe defects in short-term memory in an olfactory conditioning paradigm (Abbasi-Moheb et al., 2012).

More recently, candidates with genetic findings based on whole-exome sequencing studies have been investigated in *Drosophila*. Two loss-of-function mutations (a stop and frameshift) in the chromatin modifier *GATAD2B* were found in exome studies (de Ligt et al., 2012). These and further investigations showed that mutations in *GATAD2B* cause a recognizable ID syndrome and are associated with learning deficits and synaptic undergrowth in *Drosophila* (Willemsen et al., 2013). Thus, rare genetic findings can significantly profit from *Drosophila* models of their associated disorders.

5.2.2. Support for causality through disease-relevant gene-gene interactions

Strong support for the causative involvement of a gene in a recognizable syndrome can also be provided by demonstrating genetic interactions with an already established disease gene, or with further novel candidates identified. This can make a sufficient case to reach a genetic diagnosis, even for genetic changes identified in single patients, as has recently been demonstrated for Kleefstra syndrome. In 2006, heterozygous deletions or mutations in *EHMT1* were shown to cause this disorder (Kleefstra et al., 2006). However, defects in *EHMT1* are detected in only 25% of all patients with Kleefstra syndrome, strongly suggesting the involvement of additional genes (Kleefstra et al., 2009). By targeted approaches and exome sequencing, de novo mutations in five genes were found in four patients with the core features of Kleefstra syndrome (Kleefstra et al., 2012). These genes were *MLL3*, *MDB5*, *SMARCB1*, *NR1I3* and *MTMR9*; the latter two genes carried mutations in the same patient. Sequencing these genes in an additional cohort of 50 patients did not reveal additional mutations. Therefore, although it was immediately striking that four of these genes, like *EHMT1*, encode regulators of chromatin, the causality of these mutations remained to be demonstrated. Available information and pairwise genetic interaction experiments with *Drosophila EHMT* in the wing allowed for the reconstruction of a novel chromatin modification module with both synergistic and antagonistic interactions (Kleefstra et al., 2012). Furthermore, a genetic interaction was found between *EHMT* and the ortholog of *NR1I3*, but not between *EHMT* and *Drosophila MTMR9*. The absence of the latter interaction contributed to the individual's diagnosis of a causative mutation in the nuclear receptor gene. This shows that *Drosophila* phenotyping can inform human genetics, and facilitate clinical decision-making. Of note, clinical syndromes being genetically heterogeneous is a rule rather than an exception (Oti and Brunner, 2007). This approach is therefore widely applicable.

5.2.3. Analysis of variants

Finally, disease-relevant biological assays to directly assess the effect of a disease allele/variant are desirable (Zaghloul and Katsanis, 2010). However, constructs and transgenic flies for such an approach have to be generated, making it more time-consuming than loss or gain of function studies using available resources such as genome-wide inducible RNAi collections and overexpression stocks. Variants can be investigated in overexpression experiments, comparing gain-of-function phenotypes evoked by the wild-type allele to those caused by mutant alleles. This approach has been used in the early days in fly models of Fragile X syndrome to determine the consequence of a rare *FMR1* point mutation, I367N, located in the second KH (RNA-binding) domain (Wan et al., 2000).

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Analogous mutations introduced in either the KH1 or KH2 domain of the *Drosophila* ortholog significantly ameliorates, but does not completely suppress external and internal eye defects, demonstrating that this allele represents a partial loss-of-function allele.

Re-expression strategies to rescue a null condition with wild-type versus mutant constructs, gene replacement strategies or genome editing, are more advanced approaches that can be used to address the particular function of an observed variant of interest. Over- and re-expression approaches can express the human gene and variants, or the fly gene with introduced variant, if the affected amino acid is conserved (Coffee et al., 2012).

6. Clinical application of *Drosophila* II: toward treatment

6.1. From reversible phenotypes in models of ID to first clinical trials

ID disorders have long been thought to result from abnormal brain development rather than from acute deficits in neuronal function, because brain function in ID patients is affected at or soon after birth. They have thus been considered untreatable. However, an accumulating body of evidence demonstrates that behavioral and cognitive phenotypes in several animal models of ID disorders can be improved or restored at post-embryonic stages. Genetic and/or pharmacologic rescue in adulthood was successful in mouse models of Neurofibromatosis (*NF1*), Rett syndrome (*MECP2*), Rubinstein-Taybi (*CBP*), Macrocephaly/Autism syndrome (*PTEN*), Angelman syndrome (*UBE3A*) and Tuberous sclerosis (*TSC1* and *TSC2*), reviewed elsewhere (Castrén et al., 2012; Ehninger et al., 2008). After the early finding that statins, widely used cholesterol-lowering drugs applied to treat hypercholesterolemia, improved learning and attention deficits in the NF1 mouse model (Li et al., 2005), it took only three years until the first report of a randomized, controlled trial came out (Krab et al., 2008a). The challenges associated with translating such trials and findings to humans are discussed in (Castrén et al., 2012). Researchers of the Learning Disabilities Network (LeaDNet) and the expertise center ENCORE at the Erasmus Medical Center (Rotterdam, Netherlands) have established competence to facilitate and conduct further clinical trials in Neurofibromatosis type 1 and other "reversible" ID disorders mentioned previously, notably those associated with the Ras/MAPK and mTOR pathways (Acosta et al., 2012; ENCORE).

Despite these encouraging activities and the impact that they have on our view of the molecular nature and temporal origin of (at least some) ID disorders, work in this field is still limited. Two bottlenecks that limit the expansion of investigations in this field are: (1) identifying ID disorders in which behavioral and cognitive deficit can potentially be restored, and (2) identifying compounds that can serve as a starting point for clinical trials. Animal models, to date primarily mice, have been indispensable in gathering the knowledge necessary to initiate the first steps toward treatment described above. However, generation of mutant mouse models and breeding takes considerable time and resources, and therefore typically only up to a hand-full of compounds are tested, at least in academic settings. *Drosophila* models are ideally suited as an initial tool to determine by genetic approaches whether cognitive or other disease-relevant defects can be rescued by adult re-expression of a gene in a mutant background, and for targeted drug testing. Moreover, flies can be effectively used for drug screens, much as the traditional high-throughput screens in pharmaceutical industry that are based on in vitro cell culture or biochemical assays.

Drosophila has made a major contribution to the identification of pharmacological rescue in Fragile X syndrome. Following the mGluR theory of Fragile X, successful treatment of phenotypes including learning and memory defects with mGluR5 antagonists and lithium was first accomplished in the Fragile X fly model (Bear et al., 2004; McBride et al., 2005). In subsequent years, these compounds have proven to counteract behavioral, morphological and neurophysiological abnormalities in mutant mice and are currently being tested in clinical trials (Choi et al., 2011; de Vrij et al., 2008; Yan et al., 2005). Significant improvement of abnormal behaviors associated with the disease has been reported, at least in a subgroup of patients (Berry-Kravis et al., 2008; Jacquemont and Curie, 2011). Further studies are required, particularly with carefully assessed cognitive outcome measures.

In addition to the targeted approach, an unbiased drug screen has been carried out in Fragile X flies (Chang et al., 2008). Interestingly, three of the compounds that rescued Fragile X-related glutamate-induced lethality were positive regulators of GABA signaling. This made immediate sense, since the GABAergic system counteracts glutamatergic excitatory circuits that are overly active in Fragile X syndrome. This finding is also in agreement with previous reports on reduced expression of several GABA-A receptor subunits, consistently found in the Fragile X mouse and *Drosophila* models (D'Hulst et al., 2006; El Idrissi et al., 2005). GABA-A receptor agonists have subsequently revealed beneficial effects in mice. Ganaxolone is currently in phase II clinical trials for the treatment of epilepsy and is thus also a promising treatment option for Fragile X patients (Heulens et al., 2012).

The most recently reported successful adult rescue of a ID disorder has been accomplished in the earlier-mentioned *Drosophila* model of Kleefstra syndrome. Re-expression of EHMT from a transgene, induced in adult flies, fully restores memory (Kramer et al., 2011). The epigenetic function and genetic interactions between *EHMT* and other Kleefstra syndrome genes (Kleefstra et al., 2012) provide a lead for future drug testing.

6.2. Disease-relevant outcome measures for high-throughput drug screens

When it comes to unbiased testing of chemical compounds, *Drosophila* offers advantage over classic in vitro screens pursued by pharmaceutical companies that traditionally have a very high rate of failure late in the drug development process. Optimized lead compounds often turn out to be toxic to the complex physiology of an intact organism, or fail to exhibit the desirable characteristics for absorption, distribution, metabolism and/or excretion (ADME properties) (Pandey and Nichols, 2011). Despite their evolutionary distance to humans, it is likely that *Drosophila* models can offer complementary, if not better, information on physiological benefits and side effects of drugs than in vitro systems. As in humans, the output measure of drug screens in fly (and other animal) models is likely to be a parameter of great importance for success. The encouraging findings of the drug screen in Fragile X flies shows that even lethality can be used as read-out in a primary screen, however it should be kept in mind that not every ID gene is essential or can be sensitized to lethality as in this specific case (Chang et al., 2008). More sophisticated output measures that are directly related to the disease pathology, such as learning or memory, are desirable. This may reduce the throughput of a screen to a certain degree. However, genetic screens for learning and memory mutants have been performed for decades in *Drosophila* and novel high-throughput compatible solutions are emerging, from automated tracking of fly behavior (Dankert et al., 2009; Dubnau and Tully, 1998; Skoulakis and Grammenoudi, 2006; Valente et al., 2007) to complete (commercially available) set-ups such as Aktogen's Autojump system (Sharma et al., 2009). Testing mutant conditions in a similar paradigm, *Drosophila* light-off jump habituation has already revealed defects in non-associative learning in several *Drosophila* models of ID (Kramer et al., 2011; van Bon et al., 2013; Willemsen et al., 2013). It is to be hoped that such solutions will ultimately be

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adopted by the pharmaceutical industry in academic and private partnerships to advance drug testing for ID and related disorders.

6.3. Summary and future direction: "network medicine"

It appears unrealistic to speculate, based on a dozen studies in animal models (Castrén et al., 2012; Ehninger et al., 2008), that cognitive deficits can be potentially reversed in the majority of ID disorders. Yet, the previously listed findings are intriguing and raise hope that a significant number of such conditions exist. Likewise, it seems unlikely that cognitive impairment in humans can be cured, and we therefore believe that this term should be avoided not to raise expectations above the realistic. However, treatment that helps to improve cognitive and/or behavioral functioning, potentially in combination with environmental stimulation, would represent a major step forward for patients and their families (Castrén et al., 2012). Great effort should thus be invested in better understanding the molecular neurobiology that underlies individual or groups of ID disorders and in the translation of such knowledge to the clinics.

Despite the socio-economic importance of ID and its high prevalence in the general population, the vast majority of ID disorders are rare, in fact too rare to receive attention from the pharmaceutical industry. Therefore, it is to be expected that academic research, patient foundations and public funding have to drive progress in this field. Because of the immense costs of developing new drugs, testing/screening drugs that are already FDA-approved appears to be the most promising road forward at present. Moreover, accumulating insights into molecular mechanisms that are shared among ID disorders can be envisioned to provide the opportunity to target genetically heterogeneous patients with a common treatment. Such shared mechanisms can represent either compromised molecular networks, or more broadly compromised molecular processes. For example, among ID disorders there are at least a dozen genetic conditions that are due to mutations in components or regulators of the mitochondrial oxidative phosphorylation machinery. Oxidative phosphorylation is also affected in additional ID disorders (Haddad et al., 2013; Kriaucionis et al., 2006; van Bon et al., 2013), making approaches to improve or bypass defects in this process an attractive common theme for treatment. Molecular networks – signaling pathways or other protein–protein interaction modules – can likewise provide a common ground to target multiple conditions. The above discussed chromatin modification module underlying Kleefstra spectrum disorders and Rasopathies are only some examples. The first large-scale functional approach to ID in *Drosophila* has identified 26 phenotypically coherent modules (phenoclusters) using morphological eye and functional phenotyping. Interestingly, a validated cluster of eight ID genes has successfully predicted novel roles in synapse development for three of these genes. This cluster also includes *PTEN* and *TSC2*, two ID genes whose associated cognitive defects are reversible, making the six other genes in the same cluster (*MYO5A*, *MYCN*, *RPS6KA3*, *DMD*, *PIGV* and *UPF3B*), prime candidates for cognitive profiling, adult rescue experiments and, if successful, drug trials (Oortveld et al., 2013). *Drosophila* research is likely to contribute to such efforts.

7. From modeling monogenic forms of ID to modeling oligogenic ID and multifactorial cognitive disorders

7.1. Cognitive disorders of multifactorial inheritance

7.1.1. Emerging evidence for oligogenic ID

The identification of genes for ID has been most successful for disorders that display a monogenic inheritance pattern, many of which have been identified by genetic linkage in large consanguineous families. A large number of cases remain unsolved, but with the recent advent of exome sequencing, many cases of sporadic ID have been found to be monogenic as well, with a significant contribution of de novo mutations (Ku et al., 2013; Veltman and Brunner, 2012; Vissers et al., 2010). However, not all cases of ID display monogenic patterns of inheritance. Whereas severe de novo mutations are often limiting fecundity and thus remain rare in populations, weaker mutations can be more common and result in more complex inheritance patterns (de Ligt et al., 2012; Rauch et al., 2012).

Exome sequencing, and probably soon whole-genome sequencing, provide new opportunities – and enormous challenges – in uncovering complex patterns of inheritance underlying a fraction of ID disorders. Particularly in the mild spectrum of ID (IQ ~50–70), an oligogenic or multifactorial inheritance is likely much more frequent than currently appreciated and may account for a significant proportion of still unsolved cases of ID. For instance Bardet-Biedl syndrome, which is associated with ID, is inherited in a classic recessive pattern, but di- and trigenic inheritance has been demonstrated in some cases (Katsanis et al., 2001). In total 15 genes have been identified of which different combinations were found to segregate in families (Badano et al., 2006; Beales et al., 2003; Billingsley et al., 2010; Bin et al., 2009; Chen et al., 2011; Fauser et al., 2003; Hjortshøj et al., 2010; Katsanis et al., 2002). Other examples of ID disorders with partly complex inheritance are Dent's disease (co-inheritance of *OCRL* and *CLCN5* mutations) (Addis et al., 2013), Holoprosencephaly (*SHH* with either *ZIC2* or *TGIF1*) (Ming and Muenke, 2002; Nanni et al., 1999), Rasopathy (*PTPN11* and *SOS1*) (Fahrner et al., 2012) and other forms of ID (*MECP2* and *ATRX*; *SHANK2* and *CHRNA7*; *NRXN1* and various) (Béna et al., 2013; Chilian et al., 2013; Honda et al., 2012; Leblond et al., 2012), or ID with co-morbid ASD (*FOXP1* and *CNTNAP2*) (O'Roak et al., 2011).

These examples illustrate that genes that have so far been implicated in oligogenic ID are, with few exceptions, identical to monogenic ID genes. This suggests that it is not the genes but the strength of particular mutations that dictate whether additional factors are required for disease presentation. The above listed examples also illustrate that oligogenic inheritance is based on gene-gene interactions that occur among genes that act in the same molecular network. While striking in the gene pairs reported above, (e.g. OCRL and CLCN5 are implicated in endocytosis (Devuyst et al., 1999; Erdmann et al., 2007), ZIC2 and TGIF1 interfere with SHH signaling (Ming and Muenke, 2002; Nanni et al., 1999), PTPN11 and SOS1 both act in Ras/MAPK signaling pathways (Fahrner et al., 2012), MECP2 and ATRX chromatin proteins directly interact with each other (Nan et al., 2007), CNTNAP2 is a target gene of FOXP1 (O'Roak et al., 2011)), the extent to which these two themes will apply in general is unknown. At present the field of oligogenic inheritance is strongly dependent on hypothesis-driven data interpretation based on exactly these two themes (known disease associations and functional connections between candidates). The current view is surely highly biased.

Gene-gene interactions and additive effects are also likely to be contributing to neuropathology caused by copy-number variations, which also play a prominent role in ID. Our current knowledge of oligogenic causes of ID may only represents the tip of the iceberg. This scenario resembles the complexity and challenges of unraveling the genetics of neuropsychiatric disorders.

7.1.2. Genetic overlap between ID and neuropsychiatric disorders

Not only do some ID disorders share a oligogenic etiology with neuropsychiatric disorders, they also appear to (partly) share their underlying genetic architecture. The genetic overlap may in part be the result of the same pathogenic genes, but different genetic aberrations. While individual ID disorders are rare in the population, neuropsychiatric disorders are common. The most common cause

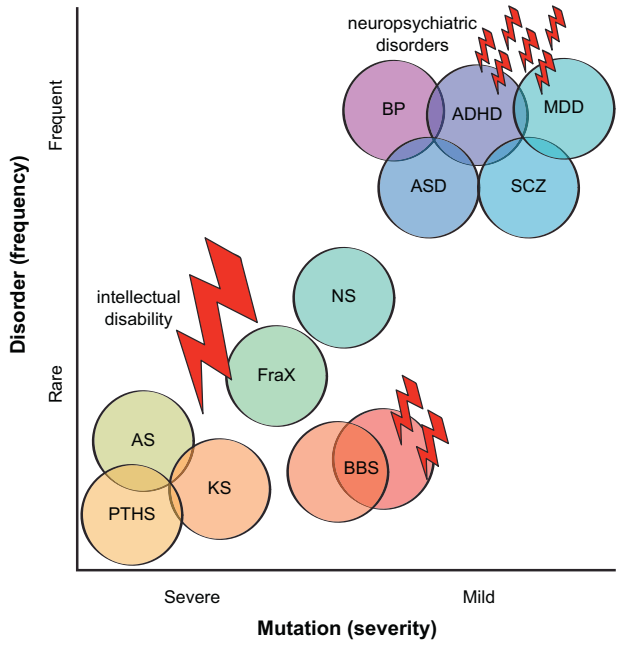


Fig. 3. Genetic overlap of ID and neuropsychiatric disorders. Monogenic disorders often arise from rare mutations that have a severe impact on gene function, while multifactorial disorders often arise from common variants with a weaker severity. PTHS, Pitt-Hopkins syndrome; AS, Angelman syndrome; KS, Kleefstra syndrome; FraX, Fragile X syndrome; BBS, Bardet-Biedl syndrome; NS, Noonan syndrome; ASD, Autism spectrum disorder; SCZ, Schizophrenia; BP, Bipolar disorder; ADHD, attention-deficit/hyperactivity disorder; MDD, major depressive disorder.

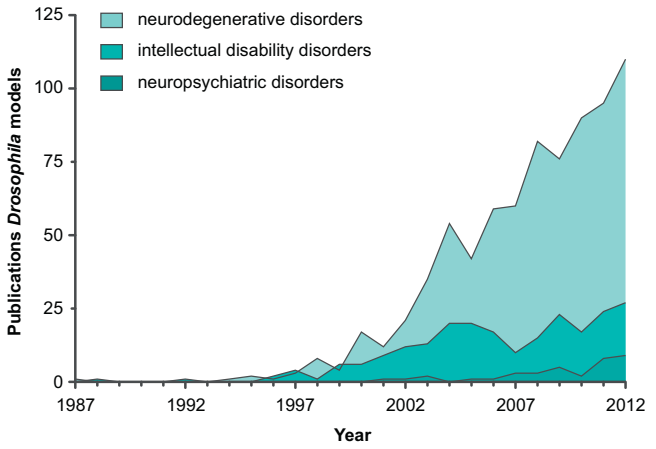


Fig. 4. Publications reporting *Drosophila* models of neurodegenerative, intellectual disability, and neuropsychiatric disorders. Publications mentioning *Drosophila* models of neurodegenerative disorders have increased linearly over the past decade, while ID models have taken off since 2007 after a preceding wave of reviews. In recent years the modeling of neuropsychiatric disorders is receiving increasing attention.

of inherited ID is Fragile X syndrome and occurs in 1/5000 males, while attention-deficit/hyperactivity disorder (ADHD) is diagnosed in 13% of boys. Interestingly, ADHD is one of the most frequently recognized disorders associated with Fragile X syndrome: 54–59% of boys with Fragile X syndrome meet diagnostic behavioral criteria for ADHD (Sullivan et al., 2006). It is intriguing to consider that similar pathways are disturbed in Fragile X and ADHD. While Fragile X is a disorder with a severe mutation in a single gene, ADHD is hypothesized to be the accumulated effect of multiple weaker gene variations (Fig. 3).

Multifactorial disorders overlap in their genetics as well, as shown for childhood ADHD and adult Schizophrenia, or suggested by the recent cross-disorder study that reported shared genetic relationship between five major psychiatric disorders (Psychiatric Genomics Consortium et al., 2013; Hamshere et al., 2013). As is recognized at the start of the DSM-5 (Adam, 2013) and by the NIMH Research Domain Criteria project (Morris and Cuthbert, 2012), psychiatric disorders should be seen as dimensional traits that share biological overlap rather than being classified as distinct groups. Environment and genetic modifiers can significantly modulate the final disease presentation.

Gene identification studies for complex genetic or multifactorial disorders still greatly lag behind classical Mendelian ones, but a strong genetic component has been established for many neuropsychiatric disorders through twin and adoption studies. For example, the heritability of the relatively common ASD is estimated to be 90% and ADHD 75–90% (Elia et al., 2012; Faraone et al., 2005; Wood et al., 2008), but the identification of genetic factors is not straightforward, resulting in the concept of missing heritability (Bailey et al., 1995; Elia et al., 2012; Faraone et al., 2005; Manolio et al., 2009; Wood et al., 2008). The mutations underlying these disorders are thought to have a small effect size and be common in the population, making their identification a challenge.

Nonetheless research has been successful in uncovering important genetic factors for presumably multifactorial traits. This applies to ADHD-like characteristics that are comorbid with a wide variety of monogenic ID disorders such as Fragile X syndrome, Tuberous Sclerosis, Neurofibromatosis I and other Rasopathies, and multigenic ID conditions such as Williams Syndrome (Curatolo et al., 2009). Additionally, autistic traits are often co-morbid with monogenic ID disorders such as Fragile X syndrome or Tuberous Sclerosis (Zafeiriou et al., 2007). Modeling monogenic ID to understand their molecular/cellular/developmental basis is thus likely to provide insights into oligogenic forms of ID and into other neuropsychiatric disorders. *Drosophila* is an excellent model system to study complex genetic interactions that underlie multifactorial disorders, because of its strength in combinatorial genetics and the wide range of established behavioral paradigms.

7.2. *Drosophila* as a model for multifactorial cognitive disorders

Modeling neuropsychiatric disorders in *Drosophila* stands now where modeling of ID was around 10 years ago: its successful application is still very limited, but it is receiving increasing attention. *Drosophila* models of neuropsychiatric disorders are increasingly discussed in the literature although these are, at present, more reviews than research papers (van Alphen and van Swinderen, 2011), just as it was the case in the first years of this millennium for ID (see Fig. 4). Many opportunities and challenges in this field lie ahead.

When variants in multiple genes are identified in oligogenic forms of ID and psychiatric genetics, it is hard to prove the causative nature of the mutations. The families often show incomplete penetrance, or multiple combinations segregate. *Drosophila* provides the excellent opportunity to perform gene-gene/protein-protein interaction studies, which can test the hypothesis of the oligogenic inheritance found in humans. Of note, such experiments can be carried out under conditions that control for genetic background and environmental factors, reducing the impact of confounding factors on the phenotype.

An example of uncovering a protein-protein interaction involved in both ID and neuropsychiatric disorder is the E3 ubiquitin ligase protein UBE3A, which is mutated in Angelman syndrome and overexpressed in 15q duplication Autism. A proteomic screen in *Drosophila* revealed that Ube3a regulates the actin cytoskeleton and neuronal homeostasis. Ube3a was found to ubiquitinate the Autism-associated Na^+/K^+ pump ATPα, stimulating new avenues of research in fly and mouse models of Angelman syndrome and Autism (Jensen et al., 2013).

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In few cases monogenic heritable neuropsychiatric disorders have been studied in *Drosophila*, such as disrupted-in-schizophrenia-1 (*DISC1*), one of the major susceptibility factors for a wide range of mental illnesses, including Schizophrenia, Bipolar Disorder, Major Depression and Autism Spectrum conditions (Sawamura et al., 2008). Expression of human DISC1 in *Drosophila* results in punctate localization of the protein in the nucleus, which was validated in primary mammalian neuron cultures. The transgenic flies display defects of sleep homeostasis consistent with altered CREB signaling. Indeed, DISC1 was found to modulate CRE-mediated gene transcription through interaction with ATF4/CREB2. Screening for genetic interactors that normalize or enhance the DISC1-induced sleep alteration would be of great interest.

Misregulation of the ortholog of the Schizophrenia susceptibility gene dysbindin (*DTNBP1*) revealed its regulatory function of glutamatergic and dopaminergic signaling, and with genetic tools exclusively available in *Drosophila*, two independent mechanisms could be identified that lead to clinically relevant behavioral phenotypes (Shao et al., 2011). First, reduced expression of dysbindin in presynaptic neurons suppresses glutamatergic synaptic transmission, which results in impaired memory. Second, reduced expression of dysbindin in glial cells causes hyperdopaminergic activities that lead to abnormal locomotion. Both behaviors can be rescued with acute genetic or pharmacological treatments in adults, suggesting that genetically relevant phenotypes in humans could be reversible.

Neuroligins have been implicated in ID and ASDs, together with several other components (Neurexins and Shanks) of the trans-synaptic signaling machinery. Deletion of *Drosophila* Neuroligin 2 impairs social interactions, alters acoustic communication signals, and affects the transition between different behaviors, resembling some core symptoms of ASDs (Hahn et al., 2013).

To facilitate translational research including drug testing, it would be an important achievement to develop or optimize behavior assays that parallels mouse and human behavior as closely as possible, yet can be conducted in higher throughput. For oligogenic disorders it will be important (compared to successful monogenic ID models) to be able to control efficiency of gene manipulation more tightly and to apply highly sensitive assays. Not necessarily, but ideally, such assays would test specific cognitive domains and recapitulate selective aspects of behavioral problems seen in the disorders. The spectrum of behaviors that can be assayed in *Drosophila* is constantly increasing. These include non-associative and associative learning and memory, social behavior, aggression, social interaction, decision-making, activity and sleep, and addiction (Anholt and Mackay, 2012; Billeter and Levine, 2013; Davis, 2011; Harbison et al., 2009; Maimon et al., 2008; Ojelade and Rothenfluh, 2009; Pavlou and Goodwin, 2013; Sokolowski, 2010). Modeling neuropsychiatric disorders in *Drosophila* is still in its infancy, but it can be anticipated that such neuropsychiatric models will 'fly off' soon.

8. Concluding remarks

With this review, we have aimed to provide an overview on the contribution that *Drosophila* research has made to date to our understanding of ID disorders and of the molecular control of cognition. Bridging the gap between fundamental and clinical research is of high important and societal significance as it can speed up scientific and medical progress. Researchers studying *Drosophila* and human biology can profit greatly from mutualisms: phenotypes of human conditions can indicate similar underlying pathologies to be investigated in *Drosophila*, while on the other hand clinical researchers can greatly benefit from the fundamental knowledge that has been acquired in the past century in *Drosophila* (Bellen et al., 2010). However, mutualism requires awareness of knowledge/publications in both fields, which is not always straightforward when genes are named differently across species. The Ensembl genome browser (www.ensembl.org) functions as a broad platform and has a great resource for quick retrieval of orthologs, using the comparative genomics pages that utilize phylogenetic trees to cluster homologs (Flicek et al., 2013). Orthologs can be found in the Ensembl gene-based display, or retrieved through a BioMart query (Vilella et al., 2009). Reversely, the database of *Drosophila* genes and genomes (FlyBase, www.flybase.org) has recently implemented an orthology section (Marygold et al., 2013) that makes it easy for *Drosophila* researchers to identify human orthologs. The FlyBase gene summary pages also contain gene function and phenotype summaries and publication lists that are informative for human geneticists and clinicians.

Here, we focused on the opportunities, not limitations, that we see and predict for *Drosophila* as a model, particularly for translational research in future medical genomics. It is not our opinion that *Drosophila* is superior to other genetic model organisms such as worm, zebrafish and mouse. On the contrary, particularly vertebrate models provide an important and indispensable bridge to translate the insights gained in *Drosophila* into future applications that will improve diagnosis, personalized patient support and treatment.

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