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Drosophila Modeling of Heritable Neurodevelopmental Disorders

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Abstract

Heritable neurodevelopmental disorders are multifaceted disease conditions encompassing a wide range of symptoms including intellectual disability, cognitive dysfunction, autism and myriad other behavioral impairments. In cases where single, causative genetic defects have been identified, such as Angelman syndrome, Rett syndrome, Neurofibromatosis Type 1 and Fragile X syndrome, the classical *Drosophila* genetic system has provided fruitful disease models. Recent *Drosophila* studies have advanced our understanding of *UBE3A*, *MECP2*, *NF1* and *FMR1* function, respectively, in genetic, biochemical, anatomical, physiological and behavioral contexts. Investigations in *Drosophila* continue to provide the essential mechanistic understanding required to facilitate the conception of rational therapeutic treatments.

Keywords

Fragile X syndrome; FMRP; Angelman syndrome; UBE3A; Rett syndrome; MeCP2; Neurofibromatosis Type 1; NF1; *Drosophila*

Introduction

Neurodevelopmental disorders (NDDs) are often characterized by defects in synaptogenesis, synaptic refinement and activity-dependent modulation, resulting in pathologically imbalanced excitatory versus inhibitory neural circuit connectivity [1]. The root of these disorders is often multifactorial, involving atypical genetic susceptibilities coupled with galvanizing environmental influences, as widely hypothesized for autism spectrum disorders (ASDs). However, a smaller number of NDDs are linked to specific, causative single gene disruptions, as in the case of *fragile x mental retardation 1* (*FMR1*) loss of function yielding Fragile X syndrome (FXS). Such monogenic disorders are readily modeled for investigations of disease etiology leading to informed, methodical development of therapeutic intervention strategies. One powerful genetic disease model is *Drosophila*, whose genome encodes 75% of human disease-associated genes [2]. The rapid generation time, comparatively low cost, and vast arsenal of genetic and transgenic capabilities available in *Drosophila* provide a proven, fruitful avenue for NDD mechanistic studies. This

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review will discuss recent *Drosophila* modeling advances for Angelman syndrome, Rett syndrome, Neurofibromatosis Type 1 and FXS.

Angelman Syndrome

With a prevalence of 1:12–20,000, Angelman syndrome (AS) is clinically characterized by severe developmental delay including marked speech and cognitive impairments, gait ataxia/limb tremulousness, altered electroencephalographic measures often with seizures, and disrupted sleep. AS patients also typically exhibit a uniquely happy demeanor and ready excitability. AS manifests with delayed, progressive onset often symptomatically noted by 3–6 months, with clinical description after 1 year [3]. The genetic disease culprit is ubiquitin ligase 3A (UBE3A), a HECT domain, E6-associated protein that drives ubiquitylation to mediate substrate degradation by the 26S proteasome. In 65–75% of AS cases, UBE3A loss of function in the maternally inherited allele, preferentially expressed in the brain [4], is cytogenetically caused by deletion events spanning 5–7Mb of chromosome 15q11–q13. The remainder of cases are linked to paternal uniparental disomy, imprinting defects and UBE3A mutations [3,5,6].

Drosophila UBE3A (Dube3a), containing a C-terminal HECT domain of 350 amino acids sharing 62% identity with human UBE3A, is ubiquitously expressed during early embryogenesis and readily detected in the developing nervous system [7]. In adult brain, Dube3a remains broadly distributed, including discernible concentration within the mushroom body (MB) learning and memory center [8]. An established model of *Dube3a* disruption demonstrates robust behavioral defects, including deficient locomotor climbing activity, circadian rhythms and long-term associative olfactory memory (Table 1) [8]. *Dube3a* variants containing human AS patient missense mutations (R626C, I925K, C55Y, T447P) mirror the behavioral loss of function phenotypes of the *Drosophila Dube3a* null, indicating strong conservation of function. The single published cellular study in peripheral dendritic arborization (DA) mechanosensory neurons shows cell autonomous neuronal architecture defects, including reduced terminal branching and incomplete receptive target field coverage (Table 1) [9].

Earlier proteomic screens of *Drosophila* brain with transgenically elevated *Dube3a/hUBE3A* identified proteins differentially driven toward polyubiquitination and subsequent degradation, including the Rho-GEF Pebble (Figure 1) [7]. Recently, the same approach demonstrated UBE3A-mediated modulation of the GTP cyclohydrolase Punch (Figure 1), an enzyme that produces the rate-limiting co-factor in monoamine biosynthesis, tetrahydrobiopterin (THB) [10]. Punch was elevated by *Dube3a* overexpression and depressed by loss of function. Accordingly, brain THB, neopterin and dopamine levels were elevated by *Dube3a* overexpression and decreased by *Dube3a* RNAi or mutation [10]. This study therefore suggests that altered dopaminergic function may contribute to AS etiology, and may shed light on symptoms associated with UBE3A copy number variants linked to ASD [11,12]. Clinically, two AS patients presenting with Parkinsonism responded positively to levodopa/carbidopa treatment [13], suggesting dopaminergic function involvement in AS. Further evidence for dopaminergic involvement in AS has been uncovered in a murine maternal loss of *Ube3a* model, in which loss of tyrosine hydroxylase-reactive neurons in the substantia nigra was linked to motor deficits [14]. Interestingly, a much earlier study in the *Drosophila* FXS disease model similarly showed Punch misregulation and altered brain dopamine synthesis, providing an intriguing FXS and AS molecular link (Figure 1) [15].

Rett Syndrome

The X-linked NDD Rett syndrome (RTT) is a leading cause of female intellectual disability, with a prevalence of 1:10,000. In RTT patients, development usually progresses normally

until 6–18 months, with subsequent, regressive loss of acquired proficiencies in expressivity and motor skills coupled with ongoing cognitive impairment, autistic behaviors and seizures [16–18]. In 90% of RTT cases, the genetic culprit is disruption of the transcriptional regulator *methyl-CpG-binding protein 2* (*MECP2*; Xq28). The remainder of cases are associated with *MECP2* duplication events [19,20] or mutations in 1) *cyclin-dependent kinase-like 5* (*CDKL5*) [21–23] or 2) the transcriptional repressor *Forkhead box protein G1* (*FOXG1*) [24,25]. These two latter molecular players colocalize with MeCP2 in nuclei of postnatal cortical neurons during maturation and synaptogenesis [21,22]. MeCP2 and CDKL5 interact directly, both *in vitro* and *in vivo* via GST-pull down and co-immunoprecipitation assays, with CDKL5 mediating MeCP2 phosphorylation [22].

Drosophila lacks an identifiable *MECP2* homolog, and thus deletion modeling cannot be pursued [26]. Instead, modeling has been based on overexpression of human *MECP2* and three RTT patient mutant alleles (R106W, R294X, and Δ166) [27–29]. The resultant eye, wing vein and locomotor phenotypes (Table 1) have formed the bases for enhancer/suppressor genetic screens [30]. In such screens, mutagenized animals are examined for second-site genetic hits that either enhance or suppress the base phenotypes, identifying genetic effectors and/or molecular interactors impinging upon the disease pathway. A candidate gene approach examining MeCP2-associating proteins linked to mammalian transcriptional repression revealed loss of function in histone deacetylase complex component *Sin3 homolog A* (*Sin3A*) acts as an enhancer, whereas loss of function in the co-repressive *SMRT-related ecdysone receptor-interacting factor* (*Smr*) and the multiple zinc finger-containing transcription factor *crooked legs* (*crol*) both act as suppressors. Suppression of wildtype *MECP2* overexpression phenotypes also occured with concomitant partial loss of function in chromatin remodeling genes (*additional sex combs (Asx), sex combs on midleg (Scm), corto* and *osa*), overexpression of the kinase *tricornered* (*trc*), and loss of the Dube3a target *pebble* (*pbl*) [30]. These findings suggest potential RTT treatment strategies targeting these MeCP2 interactors, as well as a possible molecular link between AS and RTT (Figure 1).

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) is a more common NDD (1:2,500–5,000) characterized by hallmark benign tumors on peripheral nerves (neurofibromas) and specific cognitive impairments, including difficulties with attention, executive function, language, visual perception and learning [31–33]. The genetic disease culprit is loss of *NF1* at cytological location 17q11.2, which encodes the Ras GTPase activating protein (RasGAP)/adenylate cyclase (AC) activator, neurofibromin. Disruption of *NF1* function alters the ability to restrain cellular proliferation and inhibit protein translation via the mammalian target of rapamycin (mTOR) pathway [34]. In addition, increased Ras signaling in inhibitory interneurons increases activity-dependent GABA release in the hippocampus, likely causing a number of the NF1 behavioral symptoms [35].

A *Drosophila* model based on the *dNF1* homolog (60% identity to human *NF1*) was established 14 years ago [36]. *dNF1* is involved in both Ras- and cAMP-dependent pathways interacting with the *rutabaga* (*rut*) gene encoding AC (Figure 1) [36,37]. NF1 activation of the Rut/AC pathway is an essential component of *Drosophila* learning and memory (Table 1) [38]. In these behavioral studies, a Pavlovian olfactory assay trains animals by associating an odor with an electric shock punishment, paired with exposure to another odorant without shock, thus promoting learning and memory consolidation of conditioned-avoidance [39,40]. The immediate association assay after training (~15 minutes) measures learning, whereas short-term, middle-term, anesthesia-resistant and long-term memory (STM, MTM, ARM and LTM, respectively) are tracked through training

paradigm variations (e.g. massed vs. spaced) coupled with time-dependent examination of retained avoidance behavior (e.g. STM decays within one hour of training, and LTM is detectable at 24 hours) [41]. In addition to learning and STM, *NF1* also regulates the behavioral escape flight response [36] and circadian rhythmicity [42].

More recently, introduction of human *NF1* into the *Drosophila* disease model has proved conservation of gene function. Human *NF1* deletion constructs and NF1 patient point mutations (R1391S, K1423E – reducing Ras affinity; R1276P – reducing GAP activity) yield normal learning and ARM but defective LTM in the *Drosophila* disease model, whereas the *NF1* C-terminal region differentially mediates immediate memory [43]. Most recently, *dNF1* was shown to act during the operational phase of memory acquisition, and not during stabilization and maintenance [44]. RNA *in situ* studies have revealed previously elusive *dNF1* expression within the *Drosophila* central brain, including the MB learning and memory center. Exploitation of the spatially and temporally inducible transgenic Gene-Switch system has shown that adult *dNF1* expression is sufficient to rescue 3-hour memory defects in the *Drosophila* disease model, with a selective requirement in MB α/β neurons, as α′/β′ or γ expression does not rescue behavioral defects [44]. These precise cellular delineations should aid in elucidating NF1 roles within key populations of neurons differentially involved in learning and memory.

Fragile X Syndrome

Fragile X syndrome (FXS) is the most common heritable cause of intellectual disability and ASD, conservatively affecting 1:4,000 males and 1:8,000 females, with recent estimates suggesting full mutation frequency as high as 1:2,500 [45]. The genetic culprit is most commonly an unstable CGG-trinucleotide repeat expansion in the 5' regulatory region of the *fragile x mental retardation 1* gene (*FMR1*; Xq27.3). This expansion causes hypermethylation and transcriptional silencing, resulting in loss of the mRNA-binding FMRP involved in transcript stability, trafficking and translation control. FXS is clinically characterized by delayed and depressed developmental trajectories, working memory deficits, disordered sleep, hypersensitivity to sensory stimuli, seizures, elevated anxiety, hyperactivity and 30% autism comorbidity [46,47].

FMRP loss has long been studied in both murine [48] and *Drosophila* models [49] with well-characterized defects in neuronal architecture, inappropriate activity-dependent pruning, altered neurotransmission and compromised behavioral output including disrupted circadian rhythms and defects in learning and memory (Table 1) [50–52]. *Drosophila* has a single *FMR1* gene (*dfmr1*), as opposed to the murine tripartite *FMR1*/*FXR1*/*FXR2* gene family, but unique conservation of *FMR1* function was recently established [53]. In the *Drosophila* disease model (*dfmr1* null), each human gene family member was expressed both in the germline and nervous system. Neither *hFXR1* nor *hFXR2* could restore any aspect of neuronal dysfunction, whereas *hFMR1* fully rescued all defects, including elevated translation and overelaborated architectural complexity, as effectively as native *dFMR1* [53]. In contrast, all three human gene family members were equally competent in rescuing non-neuronal *dfmr1* phenotypes in the male testes. These results show that *FMR1* maintains a unique function within neurons required for the translational control governing synaptogenesis and synaptic refinement.

Outside of these well-established functions, dFMRP was recently shown to play roles in cellular proliferation. This requirement was first evident in the germ line and during early embryonic development [54–58]. Importantly, dFMRP later also regulates the exit from quiescence and proliferative capacity of neural stem cells [59]. In *dfmr1* null neuroblasts, elevated cyclin E during cell cycle progression drives the G1/S transition and increases cell

numbers in G2/M, enhancing 5-bromo-2-deoxyuridine incorporation (an S-phase indicator). Clonal analyses using the mosaic analysis with a repressible cell marker (MARCM) technique demonstrated that *dfmr1* null neuroblasts generate more neurons than controls, which were retained into adulthood [59]. These findings suggest that FXS neurological dysfunction may be due to aberrant connectivity resulting from supernumerary neuron incorporation, in addition to known defects in synaptogenesis and synaptic pruning.

FMRP has long been suggested to act as a translational repressor, first demonstrated *in vivo* in the *Drosophila* disease model by dFMRP acting as a negative regulator of the microtubule-binding MAP1B homolog Futsch [49]. More recently, a genetic suppressor screen based on the retinal disorganization caused by *dfmr1* overexpression sought to uncover interactors in this translation mechanism and identified *poly-A binding protein* (*pabp*), *discs overgrown*/*doubletime* (*dco*/*dbt*), *oo18 RNA-binding protein 2* (*orb2*), DEAD-box, ATP-dependent *RNA helicase p62/68* (*rm62*/*Dmp68*) and *small ribonucleoprotein smD3* (*smD3*) [60]. These players are dFMRP-RNA granule components, and their overexpression inhibited dendritic branching and complexity in class IV-type sensory DA neurons, phenocopying overexpression of *dfmr1* (Table 1). With transport of such FMRP-RNA complexes achieved via microtubule motors, it is important to note that dFMRP was shown to complex with the dynein-binding Bicaudal-D (BicD) [61]. Interestingly, *BicD* mutants depress neuronal dFMRP::GFP particle abundance and motility, suggesting BicD serves to positively regulate dFMRP distribution in neuronal processes. Recently, *dfmr1* has also been shown to genetically interact with *dspastin*, encoding a microtubule-severing protein whose loss is causally linked to neurodegenerative Hereditary Spastic Paraplegia (HSP), also modeled in *Drosophila* [62]. Genetic cooperativity between *dfmr1* and *dspastin* was evident in microtubule network organization, neuromuscular junction (NMJ) synapse formation, and locomotor function (Table 1) [63]. There was an inverse correlation between dFMRP levels and mitochondrial abundance in axons and NMJ synaptic terminals, with *dfmr1* also negatively regulating the flux and processivity of mitochondrial transport. Thus, dFMRP seems to bidirectionally interact with the neuronal microtubule cytoskeleton to mediate neuronal architecture and trafficking mechanisms.

The *Drosophila* FXS model has focused particularly on neural circuits driving two disease-relevant behaviors: 1) circadian activity and 2) learning/memory [50]. In *dfmr1* mutants, the small ventrolateral clock neurons (sLN_vs) exhibit synaptic overgrowth and overelaboration in the dorsal brain protocerebrum (Table 1) [53,64–67]. Conditional reintroduction of wildtype dFMRP using the Gene-Switch system showed that transient expression during late pupal brain development (P3/4) results in striking rescue of synaptic defects. In contrast, dFMRP expression either earlier in development (larval, P1/2) or in the adult provided absolutely no benefit [64]. This study indicates dFMRP plays a temporally restricted role during late brain development, when synaptogenesis and use-dependent pruning occurs, suggesting that FXS therapeutic interventions may be most beneficially targeted towards children. As predicted by clock circuit defects, *dfmr1* nulls exhibit profound disruption of circadian rhythmicity and sleep regulation (Table 1) [68]. Reportedly, *dfmr1* null mutants display elevated sleep, including increased daytime sleep, prolonged sleep through the anticipatory period at the termination of dark phase, and increased initiation of sleep episodes, with additional impairments in waking, improper sleep rebound after deprivation and increased locomotor activity. Importantly, dFMRP expression in the MB circuit was sufficient to rescue these defects [68]. The MB is more typically studied based on its central role in olfactory learning and memory. Null *dfmr1* MB defects include axon lobe malformations, inappropriate axon midline crossing, and excessive dendritic/axonal structural elaboration (Table 1) [50,52]. Recently, MB calcium signaling has been studied using a transgenic Ca^{2+} reporter (UAS-GCaMP) [69]. Null *dfmr1* MB neurons display development stage-specific increases in depolarization-induced Ca^{2+} transients as well as

excessive Ca^{2+} mobilization from internal stores. Mutants also show decreased brain transcript levels for several Ca^{2+}-binding proteins, including frequenin1/2, calmodulin and calbindin [69]. These findings suggest that calcium signaling defects likely contribute to FXS pathophysiology underlying learning and memory impairments.

Null *dfmr1* mutants show striking defects in two commonly employed learning/memory assays: 1) Pavlovian olfactory association [70] and 2) courtship conditioning [71] (Table 1). Recent studies of the former show *dfmr1* genetically interacts with *cheerio*, the actin remodeling *Filamin A* homolog associated with Periventricular Nodular Heterotopia (PNH), with double heterozygotes defective in acquisition of protein synthesis-dependent LTM [72]. Moreover, *cheerio* expression is decreased in *dfmr1* MB after LTM-inducing spaced training, suggesting that disrupted actin organization may underlie defects in memory formation and retention. In courtship studies, recent work suggests differential requirements for *dfmr1* splice isoforms [73]. An alternatively spliced C-terminal glutamine/asparagine (Q/N)-rich domain is essential for socialization enabling normal naïve courtship levels, influences STM, and is required, but not sufficient, for LTM [73]. A separate study illustrated age-dependent cognitive impairment in *dfmr1* nulls with learning loss during training [74]. Importantly, treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium rescued both the novel learning and previously identified STM defects when introduced either during development or adulthood [74]. The fact that early developmental treatment remains effective in adults suggests age-dependent phenotypes are largely predetermined by developmental defects. Thus, early FXS interventions may be curative for otherwise maintained behavioral impairments. Conversely, it is encouraging that adult-onset intervention also successfully alleviated these behavioral phenotypes, as it suggests a retained, accessible plasticity sufficient for correction at maturity.

Finally, the *Drosophila* FXS model has even taken a step towards assaying social interaction relevant to autism (Table 1) [75]. A recent study examining exploratory behavior and interfly distances reported that *dfmr1* mutants were surprisingly hypoactive and interact less with one another than with controls, showing a decreased sociability index. Interestingly, *dfmr1* nulls interact more with a control wildtype fly than another *dfmr1* mutant [75], suggesting mutants may exhibit a form of motor dyspraxia, often described in ASD, wherein a normal receptive response to interaction is intact but internally compromised due to failure of appropriate motor output display required to maintain engagement with a partner. Such assays may aid in the detection and study of autistic phenotypes in other *Drosophila* NDD models.

Conclusions

The development of *Drosophila* NDD models has advanced considerably in recent years, including AS, RTT, NF1 and FXS models. Owing to powerful advantages in forward genetic and candidate-based screens, these models have identified key interactors of UBE3A, MeCP2, neurofibromin and FMRP (Figure 1). These discoveries shed light on molecular pathways that are viable targets for the design of pharmaceutical intervention strategies. Moreover, study of defined neural circuits and disease-related behaviors is rapidly advancing in *Drosophila* NDD models, including the circuitry and output for locomotor capacity, circadian rhythmicity and learning/memory (Table 1). These advances are now allowing for coupled developmental and cell autonomous dissection of molecular requirements. We are confident that the continuing evolution of *Drosophila* NDD models will enable understanding of the mechanistic bases underlying, and likely intersecting amongst, these devastating neurological disorders.

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Research Highlights

- 75% of identified human disease-associated genes are present in *Drosophila*.
- Angelman, Rett, Neurofibromatosis and Fragile X syndromes have been modeled.
- Genetics, biochemistry, anatomy, physiology and behavior have been examined.
- *Drosophila* has advanced understanding of *UBE3A*, *MECP2*, *NF1* and *FMR1* biology.

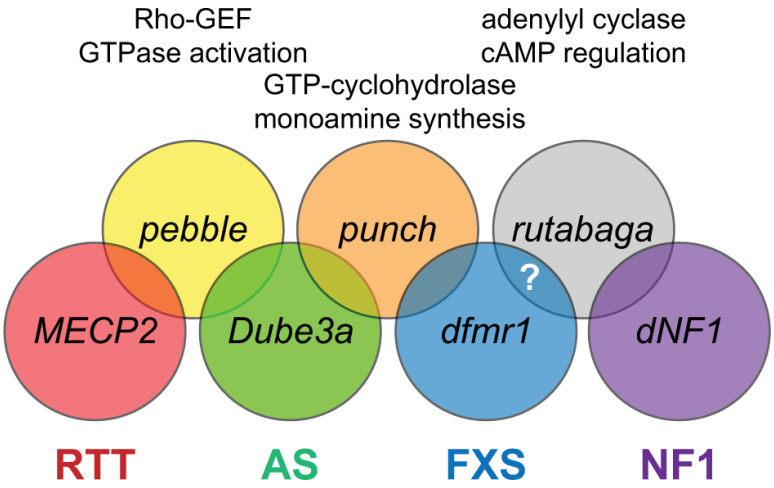

Figure 1. Molecular Intersections in *Drosophila* NDD Models

The diagram bottom row illustrates the primary genetic players in *Drosophila* models of Rett syndrome (RTT; *MECP2*, red), Angelman syndrome (AS; *Dube3a*, green), Fragile X syndrome (FXS; *dfmr1*, blue) and Neurofibromatosis Type 1 (NF1; *dNF1*, purple). The top row illustrates overlapping molecular interactors between the disease models, including the Rho-GEF *pebble* (yellow), the GTP cyclohydrolase *punch* (orange) and the adenylyl cyclase *rutabaga* (gray). Pebble is a target of the E3-ligase Dube3a, and Pebble attenuation also serves to suppress MeCP2 overexpression phenotypes. Punch is elevated by UBE3A overexpression and *dfmr1* loss of function mutations. Rutabaga has long been tied to *dNF1* and is likely to interact with *dfmr1*, as cAMP levels are altered in the *Drosophila* FXS model and FXS disease state [76].

Table 1

Comparison of Phenotypic Characterization in *Drosophila* NDD Models

The neural circuit and behavioral defects often examined in *Drosophila* NDD models are illustrated. Neural circuits with both structural and functional outputs are color-coded (locomotor (red), circadian (green), learning/memory (blue)). Model-associated defects are indicated (√), as is one stated lack of discernible defect though data was not shown (—). Absence of published studies is also duly noted (not determined; N.D.). Abbreviations: AS – Angelman syndrome, RTT – Rett syndrome, NF1 – Neurofibromatosis Type 1, FXS – Fragile X syndrome, DA – dendritic arborization neurons, NMJ – neuromuscular junction, sLN_v – small ventrolateral circadian neurons, MB – mushroom body neurons, L+M – learning and memory.

***Drosophila*-Modeled Disorder**		**AS**	**RTT**	**NF1**	**FXS**
Human Gene		**UBE3A**	**MECP2**	**NF1**	**FMR1**
Neuronal Architecture Defects	DA	√	N.D.	N.D.	√
	NMJ	N.D.	N.D.	—	√
	sLN_v	N.D.	N.D.	N.D.	√
	MB	N.D.	N.D.	N.D.	√
Behavioral Defects	Sociabilty	N.D.	N.D.	N.D.	√
	Locomotion	√	√	√	√
	Sleep	N.D.	N.D.	N.D.	√
	Circadian Rhythms	√	N.D.	√	√
	Conditioned Courtship L+M	N.D.	N.D.	N.D.	√
	Pavlovian Olfactory L+M	√	N.D.	√	√