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Review

Fruit flies and intellectual disability

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Mental retardation—known more commonly nowadays as intellectual disability—is a severe neurological condition affecting up to 3% of the general population. As a result of the analysis of familial cases and recent advances in clinical genetic testing, great strides have been made in our understanding of the genetic etiologies of mental retardation. Nonetheless, no treatment is currently clinically available to patients suffering from intellectual disability. Several animal models have been used in the study of memory and cognition. Established paradigms in Drosophila have recently captured cognitive defects in fly mutants for orthologs of genes involved in human intellectual disability. We review here three protocols designed to understand the molecular genetic basis of learning and memory in Drosophila and the genes identified so far with relation to mental retardation. In addition, we explore the mental retardation genes for which evidence of neuronal dysfunction other than memory has been established in Drosophila. Finally, we summarize the findings in Drosophila for mental retardation genes for which no neuronal information is yet available. All in all, this review illustrates the impressive overlap between genes identified in human mental retardation and genes involved in physiological learning and memory.

Introduction

Mental retardation: A clinical description. Attempts at studying the genetic basis of mental retardation date back from the beginning of the 19th century,[1] but it is only recently that a massive amount of clinical data has informed us about the molecular basis of mental retardation (MR also referred to as intellectual disabilities in the clinical literature).[2] This enormous amount of information was compiled by Dr. Victor A. McKusick and is now available freely online in the Online Mendelian Inheritance of Man (OMIM). We have indicated the OMIM reference when available. The World Wide Web address at the moment of this review is: http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim. Mental retardation affects 1–3% of the population[3-7] and consists of significantly sub-average general intellectual functioning accompanied by limitations in adaptive functioning in at least two of the following areas: communication, self care, home living, social/interpersonal skills, use of community resources, self-direction, functional academic skills, work, leisure, health and safety. The onset must be before the age of 18.[8,9] Mental retardation is described as an intellectual quotient (IQ) of less than 70. The IQ represents the relative performance of an individual (composite verbal, non-verbal) compared with the age equivalent general population performance (deviation IQ). Based on the IQ score Mental retardation can be classified as mild (50–70), moderate (35–50) or severe (less than 35). Mild mental retardation is the most common form. Males are overrepresented with a ratio of 1:1.3 to 1:1.9 due to many X-linked cases.[10] Most cases are diagnosed between the ages of 3 and 9. Various classifications have been used in mental retardation.[11] In general mental retardation is divided between syndromic and non-syndromic. Another classification relates to the inheritance pattern in multiple multiplex families: autosomal versus X-linked. X-linked mental retardation, also known as MRX, is further divided into syndromic, neuromuscular and non-specific.[10,12]

Syndromic mental retardation is diagnosed in a patient when mental retardation is associated with dysmorphic or other neurological features.[13] On the other hand, non-syndromic mental retardation is diagnosed when no hallmark features are observed in association with mental retardation.

Mental retardation's associated diseases (co-morbidity) include: cerebral palsy, epilepsy, severe hearing impairment or deafness, severe vision impairment or blindness, hydrocephalus, autism and psychiatric disorders.[5]

For many years, research on mental retardation consisted of clinical descriptions of the recurrent association of clinical signs and symptoms lumped into syndromes. Development of karyotyping through microscopic visualization of the chromosomes- allowed the linkage of mental retardation and abnormal facial or body appearance (dysmorphic features) in patients to chromosomal defects. For example, the trisomy of chromosome 21 in Down syndrome patients[14,15] represents one of many chromosomal abnormalities that can be identified on a routine karyotype analysis. In addition to variation in chromosome number, deletions, duplications, translocations and ring chromosomes can be identified by karyotyping.

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Increasing G-Banding resolution allowed identification of smaller deletions or duplications (the resolution being approximately 5 to 10 Mb). Interestingly, karyotype analysis of cells grown in growth media deficient in folate revealed the chromosomal "fragility" of the X chromosome in patients with Martin-Bell syndrome.[16] This is why Martin Bell syndrome is now known as Fragile X syndrome.

The next advance was made using fluorescent in situ hybridization (FISH). FISH surpassed the resolution of light microscopy and led to the identification of microdeletions in several syndromes. This was followed by the identification of additional microdeletions in subtelomeric regions in several patients with mental retardation.[17,18]

More recently, array-based comparative genomic hybridization (aCGH) has created a boom in the number of chromosomal aberrations discovered.[19] Multiple probes consisting of bacterial artificial chromosomes (BAC) clones of the human genome spaced by 1–1.4 megabase (Mb) are used in arrays to compare the relative number of copies for each probe in a patient compared to control genomic DNA. Targeted aCGH using more probes in regions known for human diseases have replaced the randomly distributed probes for clinical testing.[20,21] Although similar to FISH in resolution, aCGH allows for screening of more regions at once by having probes covering the entire genome. Variations in gene copy number (loss or gain) have been identified in complex cognitive disorders such as autism,[22-24] schizophrenia[25,26] and cases of mental retardation[27-32] where sporadic cases did not allow for traditional linkage-analysis based genetics.[33,34] In addition to leading to diagnosis in cases in which karyotype analysis did not pick up defects, aCGH, with its higher resolution, allows better genotype-phenotype correlation than the karyotype. Nevertheless, in clinical practice, aCGH will frequently identify deletions or duplications that involve more that one gene, making causality judgment difficult at first.

Thus far, mutations identified at the molecular level in human patients can be grouped by function: cytoskeleton modification (RhoGTPases such as GDI, PAK3, ARHGEF6, OPHN1), protein synthesis modulation (TSC1, TSC2, FMR1), chromatin remodeling (RPS6KA3, ATRX, CBP), synaptic vesicle formation and dynamics (SYN, SLC6A8, NLGN4) and transcription factors (ZNF41, ZNF81, ZNF674).[2] Importantly, these molecular functions are also key pathways in memory formation.

Several etiologies have been identified in mental retardation.[5,35,36] Despite variability in the causative lesion, however, mental retardation is associated with deficits in (i) learning new information, (ii) understanding complex information, (iii) memorizing information, (iv) transferring information from one context to another, and (v) elaborate thinking based on multiple pieces of information. This probably explains why several mutations in Drosophila genes related to human MR have been reported to cause memory defects[37-39] (Fig. 1).

Drosophila as an animal model of cognitive disorders. Despite the neuro-anatomical divergence between flies and humans, the molecular mechanisms underlying learning and memory seem to be conserved.[40,41] An emerging concept is that memory can be used as an endophenotype of cognition and its pathological counterpart, MR. More to the point, about 87% of the genes known to be involved in human mental retardation have orthologs in Drosophila.[62] As noted by Restifo,[41] this correspondence is higher than for other classes of genes.[42,43]

Drosophila is an important tool in understanding the molecular-genetic basis of mental retardation. First, Drosophila offers the possibility of studying the genetic network of a given gene. Second, it is well suited for dissection of the differential function of a gene during development and adulthood. Third, given the economy of scale, Drosophila is the perfect in vivo system in which to study pharmacological rescue. Finally, flies can be submitted to various learning paradigms (classical, operant conditioning) that can reveal task specific requirements for the gene of interest. We will now review three paradigms developed in Drosophila to study associative learning and memory: olfactory classical conditioning, operant visual conditioning and courtship memory.

Pavlovian olfactory conditioning. Initial observations and conceptualizations by psychologists such as Ribot[44] and Ebbinghaus[45] were formalized in the early 20th century with the systematic study of animal learning and memory. Learning has traditionally been identified as the early phase of new information storage. In addition, learning will sometimes be used to refer to a very early post acquisition time point for testing the new acquisition. Information stored for longer time or a later time point for testing performance related to the acquisition will be referred to as memory.[46] Memory can be then subdivided between short-term, medium term and long-term memory depending on the timing relative to the acquisition. Ivan Pavlov,[47] in particular, established experimental paradigms to study "associative" learning, a change in a behavioral response caused by the temporal association of two stimuli. Pavlov's associative learning can be considered an elemental building block of more complex forms of "contingency" learning.[48,49] In the canonical form of associative learning, an animal is presented with a neutral "conditioned stimulus" (CS), which itself does not normally elicit a behavioral response. When presentation of the CS is paired for several trials with an "unconditioned stimulus" (US), which has an inherent reward or punishment value and accordingly elicits an "unconditioned response" (UR), the CS comes to elicit a response similar to the UR. From this basic observation, Pavlov derived the general principle of stimulus substitution, which we can now think of as a basic component of more complex forms of learning. Another major behavioral psychology model for learning was defined as operant conditioning. In the operant conditioning model described by Skinner,[50] the stimulus (S) is followed by a response that is then rewarded or not (R), depending on its correspondence to the response desired by the observer. This experimental paradigm derives from the Thorndike law of effect,[51] which attributes the maintenance of a response to the effect of that response.

In Drosophila, Seymour Benzer pioneered the behavioral genetics of cognition in Drosophila.[52-54] In Benzer's laboratory at CalTech, Quinn et al.[55] were the first to show data illustrating that the Dipteran, *Drosophila melanogaster,* was capable of associating odors with footshock punishment. They used a pair of odors and subsequent reinforcement following one of the odors selectively. Using a set of tubes to which the flies had first to walk [they were attracted by a light (S)], flies were successively exposed to two odors in two different tubes (2 and 3) with footshock associated with one of the odors (R) (tube 2). Odor avoidance was then quantified by exposing the trained flies to each odor successively in the absence of footshock (tubes 4 and 5). Later, this operant odor-shock avoidance task was modified into a classical conditioning task by (i) trapping flies into

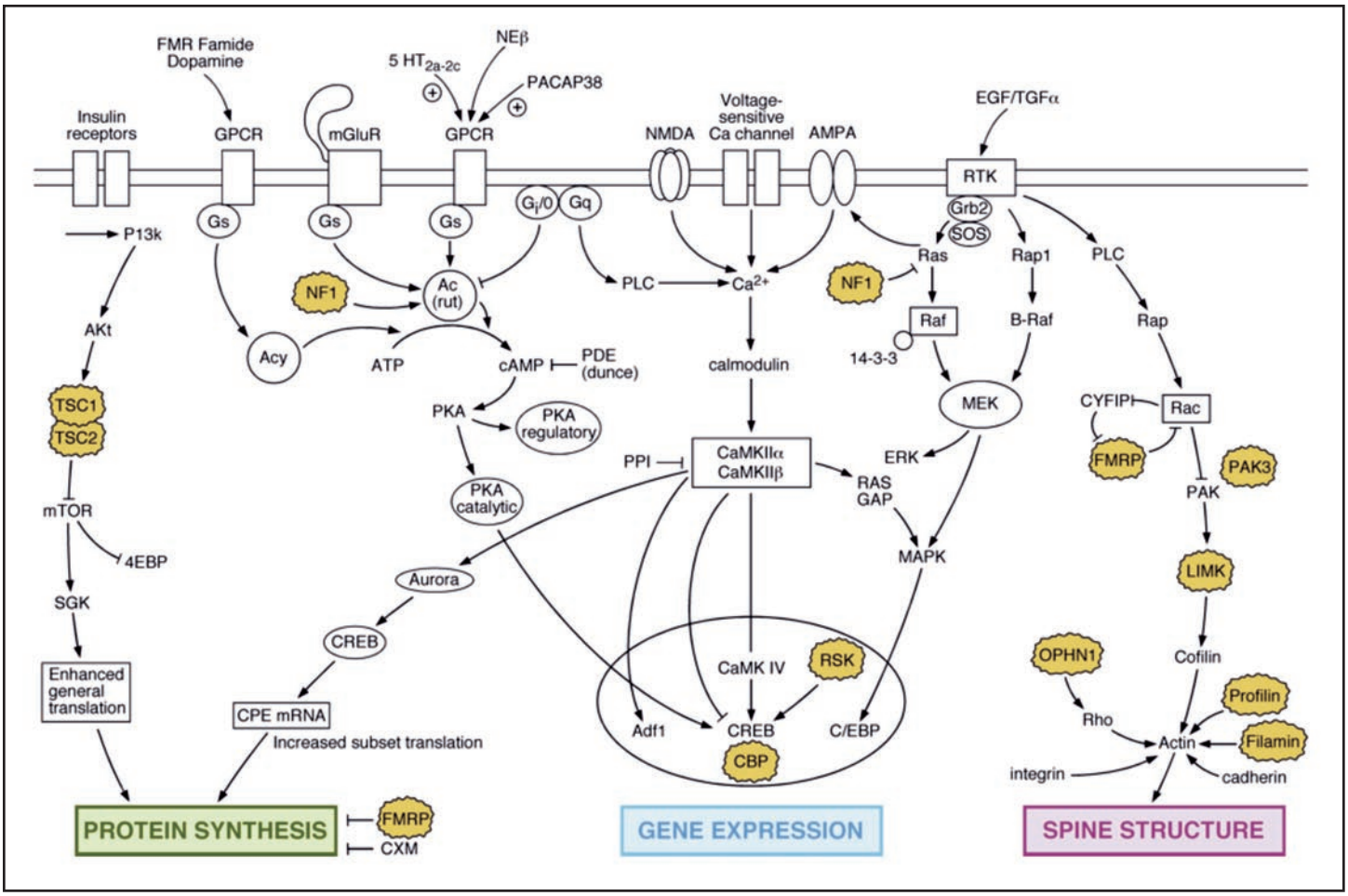

Figure 1. Memory genes identified in model systems overlap with genes identified in human mental retardation. Protein synthesis dysregulation, abnormal gene regulation and spine structure anomalies are commonly found in mental retardation. Interestingly, several of the genes involved in the signaling pathway affecting these phenotypes are responsible for learning or memory defects.

the training chamber and (ii) presenting both odors simultaneously to trained flies in a T-maze.[56] Learning retention measured immediately after one of these Pavlovian training sessions is robust. With repetitive training, flies show memory retention lasting more than a week (Fig. 2).

The development of a learning/memory task in Drosophila brought to bear the power of fly genetics to the discovery of genes involved in cognitive plasticity. It also showed that as psychologists could divide new acquisition storage into successive learning and memory phases, geneticists of memory could identify genes required for each of these phases from learning to long-term memory. The first two genes discovered as single-gene mutants, *dunce*[57] and *rutabaga*, both participate in the cyclic adenosine monophosphate (cAMP) intracellular signaling pathway (Fig. 3) and were required for learning. *dunce (dnc)* encodes cAMP-specific phosphodiesterase (PDE), an enzyme that is responsible for the degradation of cAMP,[58] while *rutabaga* (*rut*) encodes Ca^{2+}-Calmodulin sensitive adenylyl cyclase (AC), an enzyme that is responsible for the synthesis of cAMP.[59,60] Because these single-gene mutants were randomly generated and independently screened for defects in the same behavioral task (olfactory learning), these two "hits" in the same biochemical pathway most certainly underlined the importance of the cAMP pathway. Thus, cAMP signaling clearly appeared to underlie associative[60] learning in Drosophila.

In a subsequent study of these cAMP mutants, Tully and Quinn[76] obtained data that would raise an important therapeutic question: is the absolute level of a factor such as cAMP important for learning or is the critical factor its variation in relation to activity? They observed that despite near normal basal levels of cAMP in *dnc ,rut* double-mutants, the learning deficit of these flies was in fact worse than that of single-gene mutants for either *dnc* or *rut.*[56] This observation suggested that learning depends on more than the restoration of normal levels of cAMP. This finding raises challenging therapeutic concerns, as drugs that restore normal levels of a given factor may not rescue the activity-dependent variation.

Production of cAMP depends on G protein signaling.[61,62] In Drosophila, recent studies have shown learning in response to footshock (punishment) and sucrose (reward) stimuli during classical conditioning to be mediated by dopamine and octopamine, respectively.[63] Both neurotransmitters bind to G-protein coupled receptors. When ligand binds the receptor, GTP-bound Gα protein is free to interact with AC. This interaction is then terminated when the intrinsic GTPase activity of the Gα protein hydrolyzes GTP to GDP. G proteins are made of three subunits (α, β, γ), and the α

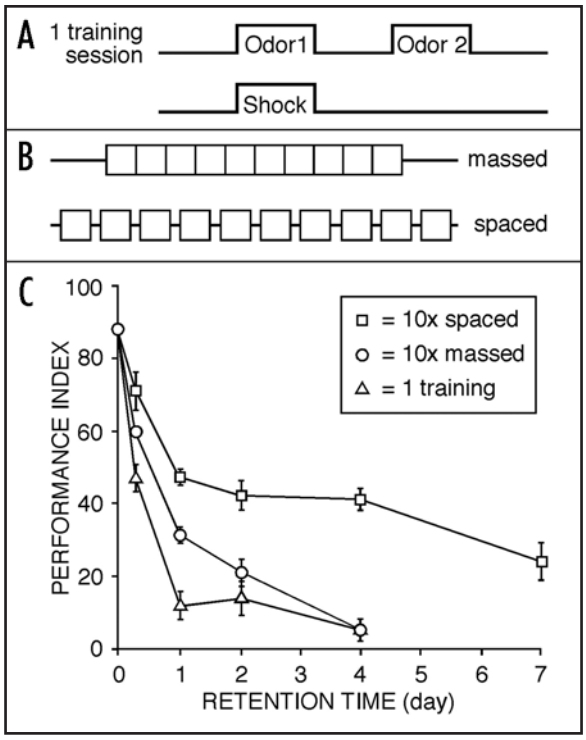

Figure 2. Classical olfactory conditioning leads to long-term memory in Drosophila. (A) Drosophila can be trained to remember the association between an odor (CS) and a footshock (US). (B) Repeated training (10 training sessions) can be performed without rest intervals (massed training) or with 15-minute rest intervals between training sessions (spaced training). (C) Performance index curve after a single training session (△) spaced training (□) or massed training (○). Single training leads to a memory that decays rapidly but persists at low levels for up to four days. Repeated training without rest intervals leads to a higher level of performance, but also lasts approximately four days. In contrast, the same amount of repeated training separated by rest intervals leads to a memory lasting for at least one week at a higher performance level (Modified after Tully et al.[75]).

subunit can either be stimulatory (Gs) or inhibitory (Gi). Connolly et al.[64] showed that constitutively active Gs disrupted Drosophila olfactory learning.

The importance of the cAMP pathway in learning was reaffirmed by the conservation of its key molecules across different species. Indeed, using a sensitization procedure in *Aplysia* for the gill withdrawal reflex in which an electric shock to the tail provokes a withdrawal of the gill, Kandel and coworkers[65,66] had uncovered the role of the cAMP pathway in learning and memory. Castellucci et al.[67,68] and Kupfermann et al.[85] established a minimal monosynaptic neural circuitry involved in sensitization of the gill withdrawal reflex. This allowed Brunelli et al.[69] to show that this simple form of learning produced an increase of the second messenger cAMP, which then led to a cAMP-dependent protein kinase A (PKA)-dependent presynaptic enhancement of neurotransmitter release at this synapse. The mechanism of increased neurotransmitter release was later shown to depend on the serotonin sensitive (S-type) K^+ channel.[70] After changes in such potassium channel currents, a given stimulus produced a stronger calcium influx in the presynaptic (sensory) neuron, thereby increasing the release probabilities of the neurotransmitter.[71]

Further dissection of the genetics of learning and memory in Drosophila uncovered even more conservation with Kandel's work in *Aplysia* (Fig. 3). In addition, behavioral genetics in Drosophila illustrated that moving downstream in the cAMP pathway correlated with performance defects downstream of learning: memory. The cAMP-dependent protein kinase (PKA) was shown to be involved in Drosophila olfactory medium-term memory. PKA is a tetramer, composed of two regulatory subunits and two catalytic subunits. In the absence of cAMP, the regulatory dimer binds the catalytic dimer and inhibits its kinase activity. In the presence of cAMP, the regulatory subunits fail to bind the catalytic dimer and thus, activate the kinase. Drain et al.[72] overexpressed an inducible transgenic construct encoding a mutant form of the regulatory subunit, which no longer was able to bind cAMP. Consequently, this transgene had a dominant-negative effect on PKA activity. When expression was induced in adult flies, a defect in memory resulted.

An important question remained, is there a link between the cAMP pathway and the gene transcription known to be required for the formation of long-term memory? Transcription inhibitors had been shown to disrupt memory in Aplysia[73] and mammals.[74] Since transcription factors (TFs) are key regulators of this process, Yin et al.[90] embarked on identifying TFs required for long-term memory. Drosophila could be trained in a single training session or could receive multiple training sessions. Tully et al.[75] showed that ten training sessions separated by 15 minutes rest intervals (spaced training) led to a non-decaying long-term memory. In the other hand, the same amount of training but without rest intervals (massed training) would lead to a memory that decayed completely after four days. Guided by early studies in Drosophila, which used mutant screens to identify the learning mutants dunce (PDE) and rutabaga (AC), by subsequent reverse-genetics methods used to dissect other components of the cAMP signaling pathway, and by the behavior paradigm for spaced and massed training, Yin et al.[76] disrupted the cAMP response element binding gene (creb) and confirmed the hypothesis that long-term memory formation would specifically be blocked. CREB is a transcription factor that binds as a dimer to a specific DNA sequence (TGACGTCA) known as a cAMP response element (CRE) that is present in the promoter or enhancer regions of CREB-regulated genes. Several genes with CRE sites are involved in neuronal plasticity. CREB has also been shown to be required in mammals. Indeed, CREB inhibition impairs memory in mice,[77-79] and injection of antisense oligonucleotides to CREB into the hippocampus or amygdala of normal rats also produces memory deficits.[78] More recently, Wagatsuma et al.[80] showed that CREB was required presynaptically for synaptic enhancement.

Impey et al.[81] showed that CREB-dependent gene transcription (as reported by a CRE-β-galactosidase transgene) was induced after protocols that yielded late long term potentiation (LTP) but not early LTP. LTP (Fig. 4) is a cellular model of learning and memory. Bliss and Lomo[82] initially described facilitation of synaptic transmission in the perforant pathway of the dendate gyrus (a region of the hippocampus) after a high frequency tetanic stimulation. LTP protocols have now been established in neurons of multiple regions of the brain. LTP is divided by N-methyl-D-aspartic acid (NMDA) receptor dependence. In the CA1 region of the hippocampus, long-term potentiation was discovered to require NMDA receptors.[83] Recently Xia et al.[84] demonstrated a role for NDMA receptors

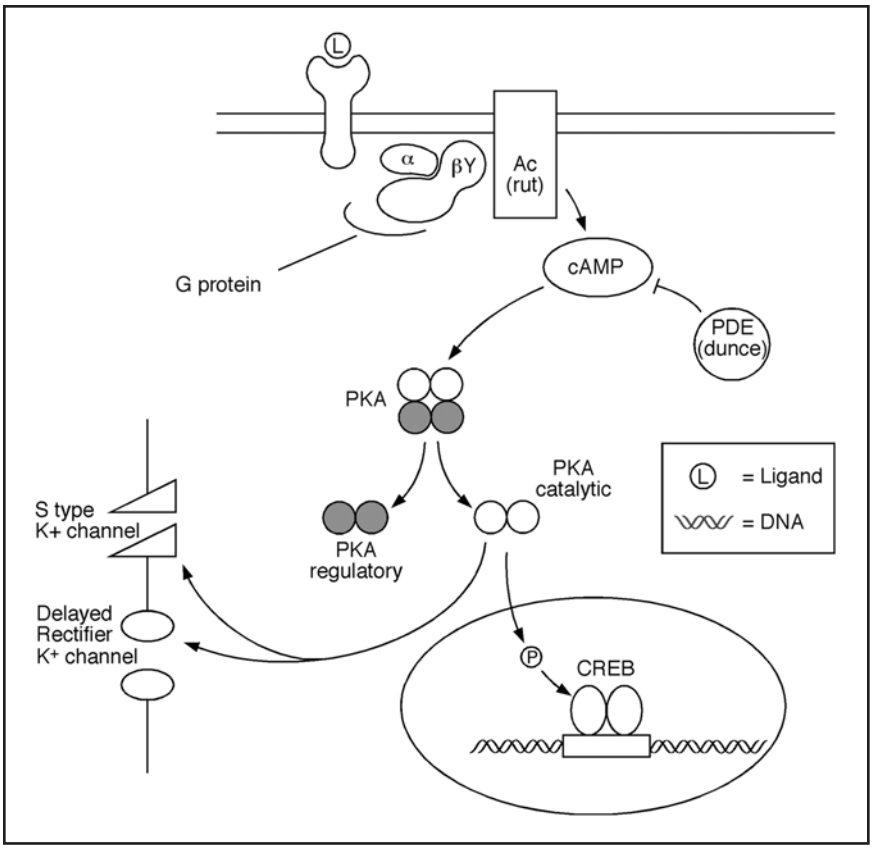

Figure 3. Learning and short-term memory depend on the cAMP pathway. After the activation of a G protein coupled receptor by a ligand (L), adenylyl cyclase (AC) synthesizes cAMP, which in turn is hydrolyzed by phosphodiesterase (PDE). The Drosophila mutants *rutabaga* (AC) and *dunce* (PDE) were among the first mutants isolated with heritable defects in learning/memory. Increased levels of cAMP lead to the activation of Protein Kinase A (PKA). After dissociation from its regulatory subunit, the catalytic subunit phosphorylates many targets: (1) potassium channels, which regulate neural activity during short-term memory phases; and (2) the cyclic AMP responsive element binding protein (CREB) transcription factor, which regulates the expression of other genes during long-term memory formation.

in Drosophila memory, again emphasizing the evolutionarily conservation in molecular mechanisms of associative learning. NMDA receptors are composed of an obligatory NR1 subunit and a variable NR2 subunit (NR2A-D).[85] Variation in NR2 subunits and subsequently the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor is associated with potentiation.[86,87] An NMDA receptor-independent form of LTP can be induced in the mossy fiber-CA3 region by glutamatergic activation of kainate receptors. This form of LTP is cAMP-dependent and appears to be presynaptic.

The converse phenomenon is known as long-term depression (LTD) (Fig. 4). LTD can be triggered by low frequency stimulation or chemically by metabotropic glutamate receptor agonists.[103] All forms of LTD seem to involve decreased postsynaptic AMPA signaling.[88,89] Long-term synaptic depression (LTD) has been associated with loss of AMPA receptors via endocytosis. The process is associated with clathrin-coated vesicles and is dependent on dynamin.

The relationship between LTP, LTD and the changes in the brains of animals involved in behavior paradigms such as conditioning in Drosophila remains incomplete. Several molecules, as well as transcription and protein synthesis, seem to be required in both models. Moreover, LTP-like changes in CA1 neurons of the hippocampus were observed following one-trial avoidance learning in rats.[90] Further studies will be required to localize LTP or LTD like process to learning and memory in Drosophila.

Operant visual conditioning. Operant conditioning is another prominent model for human learning and intelligence. Skinner[50] conceptualized this learning paradigm where an action (CS) is followed by a consequence (US), which itself serves as a positive or negative reinforcement. In flies, visual operant learning was established by Gotz[91] and then optimized by Heisenberg.[92,93] Tethered flies are placed in the center of a cylindrical arena where visual information is displayed.[94] Flies are trained to respond to the visual cues that are displayed. The fly wing movement reflects the response (CS) and is transmitted via the motor control unit that, in turn, leads to the rotation of the arena. Reinforcement (US) is produced by exposing the fly to the noxious heat generated by a light beam directed to them.

Development of multiple behavior paradigms revealed that, as in humans, not all learning/memory genes are required for all forms of leaning/memory. First, Gong et al.[95] showed that the olfactory learning mutant *dnc* has normal operant visual learning. Second, task specialization is also reflected at the neurotransmitter level using another operant conditioning paradigm. Operant conditioning has also been developing with reference to spatial localization utilizing a hot chamber and is known as operant place learning. Sitaraman et al.[96] showed that serotonin is required for operant place learning whereas dopamine is not. On the other hand Schwaerzel et al.[63] documented that aversive olfactory conditioning depends on dopamine. Third, this task specificity extends beyond molecules to anatomical circuits. Indeed, it was shown early on that the Drosophila central complex, a circular structure located in the middle of the fly brain, is required for visual learning.[97,98] In olfactory classical conditioning, most reports have identified another structure called the mushroom body as the site for memory.[99-101] An exception is the recent report by Wu et al,[99] that showed that NMDA signaling in the central complex is involved in olfactory learning. Overall, these data illustrate that in the fly as well as in humans, different forms of memory may require different genes and neuroanatomical structures.

Courtship memory. Finally, some researchers have aimed to train flies to modify an innate behavior: for example, courtship. Drosophila displays a stereotyped behavior during courtship.[102-104] Siegel and Hall[105] took advantage of this tractable behavior to study courtship conditioning. They showed that a male exposed to a female will pursue her to engage in a mating song behavior. Once mated, a female will reject the male. Typically, the male will be exposed to a mated female for 1 hour (training). During this time, he will progressively decrease the amount of time spent in courtship, resulting in suppression of courtship behavior in the last 10 minutes of the training compared to the first 10 minutes (identified as learning). Retention of this suppression can be tested when, after a given rest

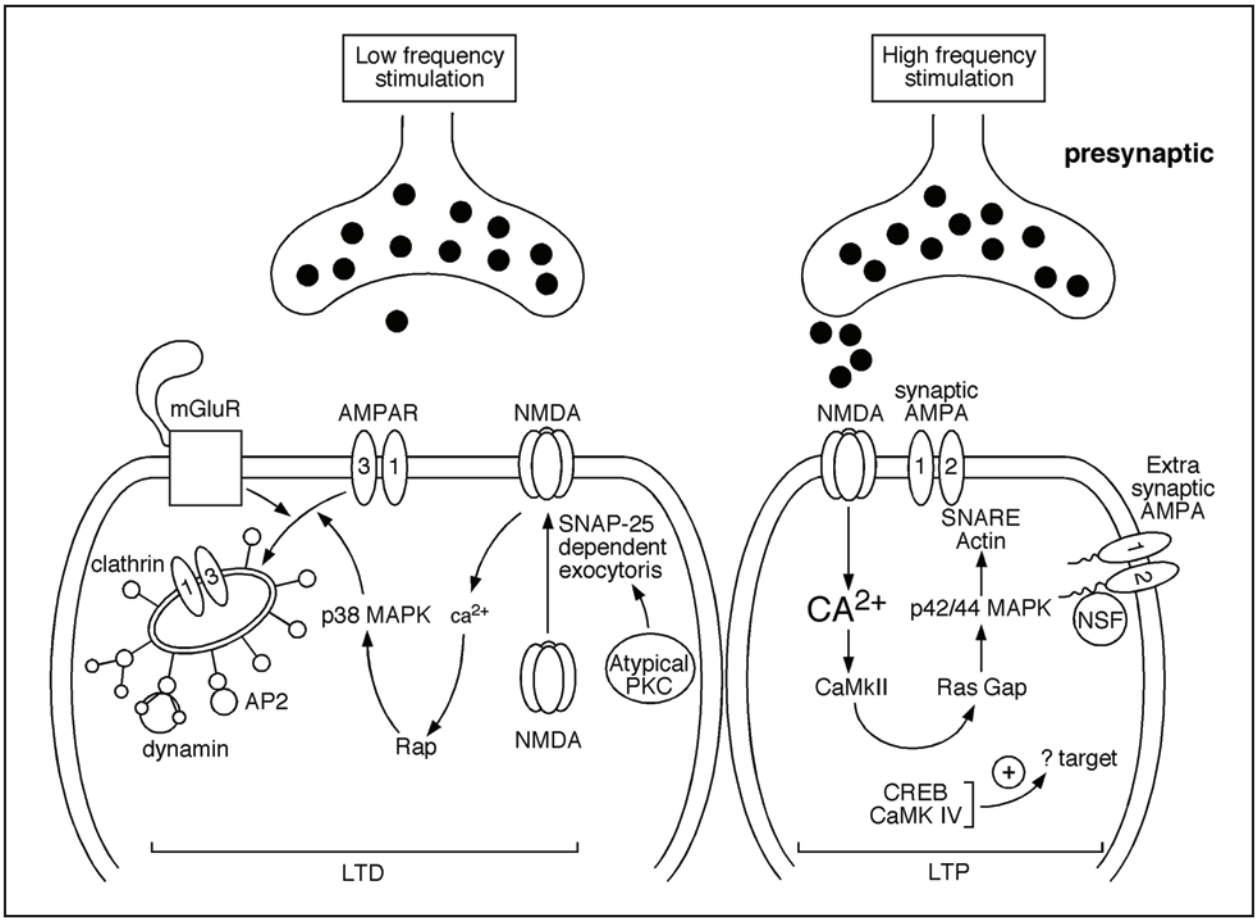

Figure 4. Long-term potentiation and depression are caused by differential signaling in response to different levels of elevated calcium. (A) Long-term depression is induced after a mild intracellular rise in Calcium in response to NMDA receptor activation. This minimal entry of calcium usually results from low-frequency stimulation of the synapse. A reduction in the number of AMPA receptors containing the subunits 1 and 3 is then observed, causing a decrease in synaptic strength. (B) In contrast, a high-frequency stimulation will lead to massive calcium entry through NMDA receptors leading to de novo AMPA receptor insertion at the synapse. The addition of these new receptors will cause a net increase in synaptic strength.

interval, the same male is exposed to another female (memory). The degree of courtship suppression is then measured.[105-107] There are multiple mechanisms involved in courtship conditioning.[106,108-110]

As for operant conditioning, courtship conditioning widens our understanding of the neuronal basis of learning and memory. Kane et al.[111] showed that males mutant for *pkc* did not decrease their courtship during the training period with a mated female, but did perform similarly to controls 10 minutes and 2 hours after training. In other words, they presented evidence of a courtship-learning deficit, but appeared to have normal memory, a phenomenon rarely seen in olfactory conditioning. Also, important plasticity molecules involved in LTP such as CAMKII and ORB, have been identified thus far as involved in courtship memory only. The inhibition of calcium/calmodulin-dependent protein kinase II (CamKII) using heat-shock induction of a peptide inhibitor leads to the disruption of courtship conditioning.[112] More recently Keleman et al.[113] showed a defect in courtship memory in *orb2* mutants, the Drosophila cytoplasmic polyadenylation element binding protein (CPEB) homolog. In summary, even in a single animal, utilization of multiple behavior paradigms is likely to help form a more comprehensive view of the ensemble of genes responsible for learning and memory.

Genes Identified in Human Mental Retardation and Shown to have a Drosophila Learning or Memory Phenotype

Albright hereditary osteodystrophy (Gs protein). Connolly et al.[64] used Drosophila genetics to test whether G protein-mediated signaling is involved in Pavlovian olfactory learning. They generated transgenic flies carrying a mutant G_s subunit that could no longer hydrolyze GTP to GDP. Thus, this mutant G protein would irreversibly bind to adenylate cyclase (AC), rendering it constitutively active. Associative learning was completely abolished in these transgenic flies. In humans, interestingly, an imprinting defect for a gene encoding G_s is known to cause Albright hereditary osteodystrophy (OMIM #103580).[114] This syndrome is characterized by short stature, obesity, round faces, subcutaneous ossifications, brachydactyly, other skeletal anomalies and mental retardation.[115]

Mental retardation autosomal recessive-MRT1 (Neurotrypsin). Mutations in the human PRSS12 gene, which encodes the protéase Neurotrypsin, lead to non-syndromic autosomal recessive mental retardation (OMIM #606709).[116] Didelot et al.[37] identified long-term memory defects in flies with reduced expression of *tequila*, the Drosophila ortholog of Neurotrypsin. Importantly, this was the

first demonstration that a gene involved in human mental retardation has an acute role in memory formation. Furthermore, Didelot et al. were able to localize the requirement for *tequila* expression to the mushroom bodies. The role of proteases in neuronal migration, axon outgrowth[117,118] and synapse elimination has been reviewed by Molinari et al.[119]

Fragile X mental retardation syndrome (Fmr1). Several mental retardation conditions due to single gene defects have been identified to date. Fragile X mental retardation syndrome (OMIM # 309550) is the most common cause of single gene mental retardation in males (1/4000) and is a leading cause in females (1/8000). Mental retardation (MR) is moderate to severe.[120,121] Fragile X syndrome is associated with autism and epilepsy. Initially localized to the X chromosome based on karyotype defects,[122] the precise molecular lesion was later linked to a trinucleotide (CGG) repeat expansion above 200.[123,124] Expansion in the 5' untranslated region (UTR) of *Fmr1* leads to DNA methylation and lack of FMR protein (FMRP) expression.[125] In some cases, however, deletions[126,127] and even point mutation[128] are responsible for Fragile X syndrome. Age-dependent changes in the FMR phenotype and developmental effects have also been observed.[129,130] Although caused by a single-gene mutation, understanding the cognitive phenotype in Fragile X syndrome is complicated because of the multiple protein-protein and protein-mRNA interactions of FMRP.[131-133] Biochemically, FMRP has been shown to be involved in translational control.[134,135]

Drosophila has a homolog of Fmr1 known as dFmr1.[136] At the protein level, conservation of important domains varies between 25 and 75%.[137] Initial studies of Fragile X mutant flies identified a defect in circadian rhythm, a debilitating symptom commonly found in patients with Fragile X.[138,139] The overlap in neuronal dendritic spine abnormalities is yet another example of similarities between flies[132,140] and humans in the Fragile X mutant phenotype.[141-143] Fragile X patients[144,145] and FMR1 knock-out (KO) mice display spatial learning deficits.[146-148] Also, enhanced hippocampal long-term depression (LTD)[149] and defective cortical long-term potentiation (LTP)[150,151] are found in FMR KO mice.

In Drosophila McBride et al.[152] have shown that the Drosophila 3-hour courtship conditioning memory is impaired in *dFmr1* mutants. Moreover, they demonstrated a pharmacological rescue of the phenotype using the metabotropic glutamate receptor 5 (mGluR5)-specific antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP) and Lithium. We have shown that the defect in long-term memory (assessed at one day) is specific to memory formed after spaced training.[38] Indeed, 1-day memory performance after massed training was normal in *dFmr1* mutants. Spaced training induces protein synthesis-dependent memory in addition to a protein synthesis-independent memory, whereas massed training involves only protein synthesis-independent memory.[75] We therefore reasoned that a disruption in the control of protein synthesis in *dFmr1* mutants was causing a negative impact on memory and rescued it with use of protein synthesis inhibitors such as cycloheximide and puromycin.[38] We also demonstrated an interaction between FMRP and *staufen* in long-term memory measured at 1 day. Staufen was initially identified in translational control in the fly oocyte. As *staufen* was previously identified in our laboratory in a screen for genes required for long-term memory formation,[153] the genetic interaction between dFmr1 and staufen in LTM behavior reinforces the link between physiological memory genes and mental retardation genes. It also established for the first time a role at the cognitive level for the interaction between *staufen* and *dFmr1*. This observation was also supported by the previous co-localization of Staufen and dFmr1 observed in subcellular structures involved in mRNA modulation known as processing bodies (p-bodies).[154]

Consistent with the idea that RNA processing, and RNA interference in particular, is involved in memory, FMRP was shown biochemically to be part of the RNA-induced silencing complex (RISC)[155] and we found that dFMRP was acutely required in long-term memory.[38] In addition, Drosophila *armitage* mutants were shown to be defective in one-day memory after olfactory spaced training.[156] *Armitage* codes for a silencing defective 3 (SDE-3) class RNA helicase responsible for the unwinding of double stranded RNA (dsRNA) and its subsequent loading onto RISC.[157]

RNA interference can be further divided in several pathways. We sought to identify modifiers of dFmr1 among known RNA interference molecules. Jin et al.[158] had demonstrated a genetic interaction between *dFmr1* and *argonaute 1 (AGO1),* an important molecule for RNA interference. Indeed, flies heterozygous mutant for *dFmr1* and AGO1 displayed abnormal morphology of neuro-muscular junctions. We identified an interaction between *ago1* and *dFmr1* in long-term memory specifically.[38] In addition, we rescued the memory deficit observed in *ago1*/+, *dFmr1*/+ double heterozygous mutants using protein synthesis inhibitors such as cycloheximide and puromycin.[38] Taken together, these results suggest that *dFmr*1 mutants may be defective in a*go1*-dependent translational control. These mutants would have excessive baseline general protein synthesis that could be occluding the normal activity-dependent protein synthesis required for memory. A similar rescue of memory using another protein synthesis inhibitor, rapamycin, was obtained in a mouse model of tuberous sclerosis of Bourneville, in which there is also an excess of protein synthesis.[159] Since both Fragile X and Tuberous sclerosis syndrome also cause autism, these findings suggests that social cognition and autism may be related to protein synthesis control.[160]

Kelley et al.[161] expanded on previous reports of altered cAMP levels in Fragile X patient cells[162,163] by showing that the induction of cAMP is reduced in humans, mice and flies defective in Fragile X. Another connection with early research on learning and memory was the observation that the circadian rhythm disruption may be linked to abnormal CREB level fluctuations in Fragile X Drosophila mutants.[138]

Neurofibromatosis type 1 (NF1). Neurofibromatosis type 1 (OMIM #162200) is clinically characterized by multiple tumors (optic neuroma, schanomas, plexiform neurofibroma), skin anomalies (café au lait spots, axilary freckling), increased head size (macrocephaly) and learning disability.[164] At the molecular level, the Neurofibromin protein contains a Ras-specific guanosine triphosphatase-activating (Ras-GAP) domain. GAP proteins enhance the intrinsic hydrolytic activity of Ras proteins, enhancing the GTP (active) to GDP (inactive) transition. Persistent activation of Ras has been suggested as an explanation for multiple tumors observed in NF1 patients.

nf1 mutant flies have decreased body size and deficits in Pavlovian olfactory learning.[165] Interaction between NF1 and the cAMP pathway[165] is suggested by rescue of *nf1* mutant body size and learning defects with PKA overexpression. In addition, mutation

of *nf1* blocks the mammalian pituitary adenylate cyclase activating peptide (PACAP)-induced enhancement of potassium current at the larval NMJ.[166] PACAP stimulates cAMP synthesis via two different pathways: the *rutabaga* AC and the Ras-Raf pathway. Moreover, Quinn et al.[167] identified a PACAP homolog mutant, *amnesiac*, with normal learning but defective medium-term memory.[168,169] Further biochemical dissection of the NF1 protein showed that a region outside of the Ras-GAP domain is responsible for the growth defect present in NF1 mutants.[170] Recently, Ho showed that the one-day memory defect is associated with the altered NF1-regulated Ras activity and not cAMP levels.[171] In summary, NF1 defects in learning and memory seem to be linked to dysfunction in different signaling pathway by different domains within the NF1 protein.

Angelman syndrome (UBE3A). Angelman syndrome (OMIM #105830) was described in 1965 in children with severe mental retardation, fits of laughter, epilepsy, ataxia and specific dysmorphic features. A special behavioral characteristic was the appearance of a happy demeanor, which led to them being described as "happy puppet". The patient also presents microcephaly, especially when the disease is related to a deletion in the 15q11.2-q13 region. Patients lack the maternal active copy of the ubiquitin ligase E3A. UBE3A is a type of E3 ubiquitin-protein ligase characterized by a HECT (homologous E6-AP carboxyl terminus) domain. The HECT domain is required to transfer ubiquitin to proteins targeted for degradation via the ubiquitin-proteosome pathway.[172] Mice with maternal inactivation of the Ube3a genes present impaired long-term potentiation and a deficit in context-dependent fear conditioning one day after training.[173] In addition to UBE3A, the ubiquitin proteosome pathway has been linked to memory in the case of proteosome-dependent degradation of Armitage (a protein required for olfactory memory).[156]

We observed that the Drosophila *dube3a* mutants showed a significant defect in one-day performance after spaced training when compared with appropriate genetic controls.[39] One-day memory after massed training was similar to controls. In addition to supporting a role in long-term memory as shown previously in mice,[173] the specific impairment of memory after spaced training (which is transcription and translation-dependent[75]) in *dube3a* mutant flies suggests that the role of UbeE3A in cognition could be related to transcriptional regulation[174] and/or protein degradation.[175]

Periventricular nodular heterotopia (Filamin A). Filamin A mutations have been found in patients with periventricular nodular heterotopia (OMIM #300017), and these patients suffer from cognitive dysfunction and epilepsy.[176,177] The Filamin A gene is involved in actin cytoskeleton remodeling.[178,179] Filamin A is expressed in neurites of embryonic rat hippocampal neurons.[176] Disruption of Filamin A impairs neuronal migration, probably because ligand binding no longer induces actin reorganization.[176,180] Drosophila's ortholog of Filamin A is cheerio. A behavioral screen in our laboratory identified joy, a mutant which carries a P-element insertion within cheerio.[153] Joy has normal immediate learning but is defective in one-day memory after spaced training. It remains unclear to which extent this phenotype is developmentally regulated.

Coffin-Lowry syndrome (RSKII). CREB is thought to be constitutively bound to DNA but inactive until phosphorylated. Phosphorylation at serine 133 promotes transcription.[181] Several kinases are known to target serine 133 of CREB for phosphorylation, among these are PKA, CaMKIV and MAPK-activated ribosomal S6 kinase (Rsk). In humans, the mutation of Rsk 2 is known to cause Coffin-Lowry syndrome (OMIM#303600). This syndrome is characterized by mental retardation, a peculiar pugilistic nose, large ears, tapered fingers, drumstick terminal phalanges on x-ray and pectus carinatum. Four RSK isoforms are present in humans, whereas Drosophila has only one. Putz et al.[182] used visual operant and classical olfactory conditioning to identify the requirement for Rsk in each paradigm. They showed that Drosophila null for rsk were deficient in olfactory learning, whereas rsk P-element derived hypomorphic mutants were defective in visual learning. The N-terminal region of the protein was required in both paradigms.

C.A.D.A.S.I.L. (*Notch*). Mutations in *Notch 3* have been identified in cerebral arteriopathy, autosomal dominant with subcortical infarct and leukodystrophy (C.A.D.A.S.I.L.) (OMIM #125310). CADASIL is clinically characterized by recurrent migraine and strokes, progressive focal neurological signs (pseudobulbar signs), seizures and dementia. *Notch* is another "developmental gene" previously known to be involved in cellular differentiation during neuronal development that serves both as a transmembrane receptor and a transcription factor. It has been documented that *Notch* in Drosophila has a role in long-term memory formation.[183] Presente et al.[183] used a temperature-sensitive allele to limit the possible contribution of developmental defects to the phenotype observed. Similarly, Costa et al.[184] showed that *Notch* mutant mice display memory defects in a water maze task. Mice expressing a *Notch* antisense transgene exhibit normal development but have a decreased level of LTP.[185]

Notch is also connected with dementia by its interaction with its ligands Delta, Serrate or Lag-2 (DSL). Notch is known to be cleaved by a furin-like convertase and to insert in the cellular membrane. Binding with DSL then triggers a gamma secretase-dependent cleavage, thereby releasing an intracellular fragment that then translocates to the nucleus. Presenilin, for which mutations have been identified in some patients with Alzheimer's disease, is a component of gamma-secretase.[186] Furthermore, mutations in presenilin were shown to abolish Notch signaling by blocking its access to the nucleus.[187]

MRX58 (Integrin). Mutations in transmembrane 4 superfamily 2 (TM4SF2) have been linked to X-linked mental retardation 58 (MRX58) (OMIM # 300096), which was initially reported in an Austrian family.[188] A behavioral screen for long-term memory mutants in our laboratory identified an allele of a tetraspanin gene (TSp42Ef).[153] TM4SF2 is a member of the Tetraspanin family of genes, which is involved in the β-1-integrin pathway. β-1-integrins are usually localized presynatically and join α-integrin that are localized post-synaptically. Alpha-integrin mutants have impaired short-term memory in Drosophila.[189]

CRASH syndrome (L1-CAM). L1 cell adhesion molecule (L1-CAM) is involved in mammalian memory formation.[190,191] L1 was originally identified in patients with X-linked hydrocephalus. Further investigation of these patients has revealed a more complex affliction known as CRASH syndrome (OMIM #308840). CRASH disease stands for corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia, and hydrocephalus.[164]

Neuroglian is the Drosophila homolog of L1-CAM. Mutations in *neuroglian* (*nrg*) were identified in a screen for learning and

locomotor activity.[192,193] De Belle and Heisenberg showed that *nrg* mutants, referred to as *central brain deranged*, had neuroanatomical defects in the central complex. Liu et al. showed that short-term visual memory was defective in *nrg* mutants.[194] Mutants for nrg^{849} have decreased microtubules at the active zone of giant neurons, resulting in an abnormal synaptic terminal morphology. In addition to these structural defects, these mutants also display functional defects in synaptic responses.

Down syndrome (DYRK1A). Trisomy of chromosome 21 leads to Down syndrome (OMIM #190685), which is characterized by mental retardation and dysmorphic features. Down syndrome is the most common cause of mental retardation.[195] Most patients (90–95%) become fully trisomic because of a non-disjunction in meiosis of chromosome 21. Some patients (2–4%) will have mosaicism for trisomy 21. Since chromosome 21 contains an estimated 225 genes, it is complicated to draw genotype-phenotype correlations.[196] Nonetheless, some patients presenting with Down syndrome have partial chromosome 21 trisomy by inheriting translocations (2–4%).[197] This partial trisomy has allowed the definition of a "critical region". The dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) is encoded by a gene located in the critical region of chromosome 21 for Down syndrome (OMIM #190685).[198] DYRK1A phosphorylates multiple targets: signal transducer and activator of transcription 3 (STAT3), the ε subunit of eukaryotic initiation factor 2B (eIF2Bε), Tau, forkhead family transcription factor (FKHR), dynamin, glycogen synthase and cyclin L2.[199]

The Drosophila ortholog of DYRK1A is known as *minibrain* gene (*mnb*).[200] Heisenberg has shown that defects in *mnb* lead to olfactory learning defects but preservation of learning of color discrimination or visual based operant learning.[201] *mnb* is involved in postembryonic neurogenesis and mutations are associated with an abnormal spacing of neuroblasts.[202] This results in reduced optic lobe and central brain hemisphere size. These results suggest a role for *minibrain* in cognition but more work is required to link an increase in DYRK1A copy number to the cognitive symptoms in Down syndrome.

Epilepsy, X-linked, with variable learning disabilities and behavior disorders (Synapsin). A mutation in Synapsin was identified in a family with epilepsy and learning disabilities (OMIM* 313440).[203] Synapsin enhances the GTPase activity of Rab3a and Rab3a recruitment to the synaptic vesicle membrane.[204,205] In Drosophila *synapsin* mutants, Godenschwege et al.[206] showed defects in learning and memory and faster habituation in the olfactory jump response.

Mice mutant for Synapsin 1 have delayed synapse formation.[207] Chin et al.[208] also demonstrated abnormal synaptic vesicle clustering in these mice. In addition to these developmental abnormalities, they identified abnormal recovery after high frequency stimulation in *synapsin* adult mice.

Genes Identified in Human Mental Retardation and Shown to have Neuronal Phenotype Other than Memory in Drosophila

In this section, we review genes involved in human mental retardation but for which we could not identify in the literature a cognitive phenotype.

Walker-Warburg syndrome (POMT1). Protein-O-mannosyl transferase 1 (POMT1) is involved in Walker-Warburg syndrome (OMIM #236670) and limb girdle muscular dystrophy type 2K (OMIM #609308).[209] Walker-Warburg is characterized by mental retardation in addition to cobblestone lissencephaly, congenital muscular dystrophy, cataracts and other anomalies.[210] Limb-girdle muscular dystrophy 2K is associated with mild proximal weakness starting at an early age, mild calves or thigh pseudohypertrophy, elevated CK, microcephaly, and mental retardation.[211] The location of the mutation within the gene influences the phenotype.[212]

The Drosophila ortholog of POMT1 is encoded by *rotated abdomen (rt).* Drosophila mutants are viable but present a clockwise rotation of the abdomen, which causes mating defects.[213] Recently, the Drosophila *rt* mutation was identified in a screen for synaptic mutants in Drosophila.[224] The mutants displayed decreased neuromuscular synaptic release probability and changes in glutamate receptor subunit composition. Indeed, DGluRIIA is absent and the levels of DGluRIIB are decreased in flies containing a P-element insertion within *rt*. The decreased probability of release seems to be due to a defect in the glycosylation of Dystroglycan.

Down syndrome (SIM2). As noted above, trisomy of chromosome 21 leads to Down syndrome (OMIM #190685), which is characterized by mental retardation and dysmorphic features. SIM2 is one of the genes in a critical region of chromosome 21 for Down syndrome.[210] The ortholog of SIM2 is named Single-minded (Sim) in Drosophila. Sim is involved in neurogenesis and midline cell fate determination.[215] Sim is a transcription factor that is part of a family that contains a domain similar to the basic helix-loop-helix (bHLH) motif. Another member of this family is the circadian rhythm protein Period.[216] Sim activates midline gene transcription [217] while repressing the lateral gene expression.[218,219] After heterodimerization with Tango, Sim migrates to the nucleus where it binds the CNS-Midline Element sequence, which leads to *hedgehog* transcription.[220] The role of Sim is not limited to the brain, it is also required in eye development.[221] Locomotor deficits have been observed in *sim* mutants. Interestingly, the mutants can only walk in circles.[222] This behavioral shortcoming is attributed to a defect in the central complex.[223,224]

Down syndrome (Dscam). Another gene previously identified as present in three copies in Down syndrome patients with congenital heart disease is *Dscam.*[225] *Dscam* is a member of the immunoglobulin superfamily and constitutes a new class of neural cell adhesion molecules.[226] The fly ortholog was identified[227] and shown to be able to generate multiple isoforms by alternative splicing. Hattori et al.[228] engineered a *Dscam* gene that could not be alternatively spliced and therefore produced a single isoform. They observed that the embryonic ventral cord wiring was severely disrupted. Also, they showed abnormal crossing of the B-lobe of the mushroom body across the brain midline.

α-thalassemia/mental retardation syndrome (XNP/ATRX). A mutation in the XNP/ATRX gene causes mental retardation syndromes associated with facial dysmophic features, urogenital defects and α-thalassemia (OMIM #301040).[239] XNP/ATRX encodes an SNF2 family zinc-finger ATPase/helicase protein.[240] It has been shown to be involved in DNA methylation,[240] chromatin remodeling,[241] transcription,[242] the cell cycle and apoptosis.

dATR-X is the Drosophila homolog of the disease gene causing α-thalassemia/mental retardation X-linked.[232-238] Sun et al.[249] identified dATRX as an enhancer of *jing* in axon scaffolding formation by glia cells. Lee et al.[240] isolated another Drosophila homolog

of XNP/ATRX that they named dXNP, and they showed that ectopic overexpression of dXNP in the Drosophila eye led to apoptosis. The fly and human proteins are most conserved in the helicase and SNF2 domains.[239] Sun et al.[239] showed that in flies, dATRX is expressed both in neurons and glial cells. In addition, overexpression of dATRX led to thinner longitudinal connectives and reduced spacing between the anterior and posterior commissures. dATRX RNAi expression in neurons results in neuronal crossing and disruption of the longitudinal fibers.

Mental retardation, hypopigmentation (WOC). Van der Maarel et al.[241] reported the case of a female patient with mental retardation, hypopigmentation over the abdomen and scoliosis that presented with a mutation in the 5' UTR region of the zinc finger gene ZNF261. ZNF261 is part of a polypeptide complex also containing the histone deactylase 1 and 2 (HDAC1/HDAC2).[242] *without children (woc)* encodes the Drosophila homolog of ZNF261. Mutant males and females are sterile.[243] Raffa et al.[244] showed that Woc protein is localized to telomeres and serves as a transcription factor with a telomere-capping function. Raffa et al. also showed that *woc* mutants have frequent telomeric fusions in Drosophila brain cells.

Genes Identified in Human Mental Retardation and Shown to have Extra-Neuronal Phenotype in Drosophila

In this section, we review genes involved in human mental retardation but for which we could not identify in the literature a cognitive or neuronal phenotype.

Cornelia de Lange (NIPPEDB). A mutation in Nipped-B-Like (NIPBL) (OMIM #122470) has been identified in approximately 50% of Cornelia de Lange syndrome patients.[245] More rarely, mutations in SMC1L1 are identified (OMIM #300590).[246] Cornelia de Lange syndrome (CdLS) is characterized by synophrys, variable mental retardation, growth retardation, hirsutism, and upper limb reduction defects that range from subtle phalangeal abnormalities to oligodactyly.[247] NIPBL is involved in sister chromatid cohesion via the cohesin complex, and is also involved in long range enhancer-promoter interaction.[248] Interestingly, a mutation in the cohesin complex (OMIM #610759) has been identified in a third Cornelia de Lange group. Recently Gause et al.[249] have shown in Drosophila that NIPBL is co-localized with cohesin in somatic and meiotic cells. Furthermore, in meiotic cells, it is co-localized with the synaptonemal complex.

Rubinstein-Taybe syndrome (CBP). Finally, we return to the cAMP pathway and CREB. Once phosphorylated at serine 133, CREB recruits CREB binding protein (CBP). CBP is a histone acetyltransferase (HAT) that leads to chromatin opening, exposure of DNA to polymerase II and transcription. Deletions within CBP are responsible for Rubinstein-Taybi syndrome (RTS) (OMIM #180849). This syndrome is characterized by mental retardation (IQ average 51), seizures (23% of patients), microcephaly and various dysmorphic features, such as downward slanted palpebral fissures, hypoplastic maxilla with narrow palate, small mouth opening, broad thumb with radial angulations and broad great toes. Oike et al.[250,251] first established a mouse model of RTS and showed defective long-term memory in passive avoidance and fear conditioning tasks. Bourtchouladze et al.[252] went on to show that mutant CBP mice have a selective deficit in long-term memory but normal short-term memory. In accordance with the previously established cAMP pathway, phosphodiesterase inhibitors ameliorated this long-term memory defect, presumably by increasing cAMP levels and CREB phosphorylation after training. Importantly, this outcome in the mouse model of RTS suggests that (i) some forms of mental retardation result from functional (biochemical) rather than structural (developmental) deficits in brain function, and (ii) the former will respond to traditional drug therapy.[253] The role of CBP has not been studied for memory in Drosophila, but Jung et al.[254] recently showed that CBP mutant flies experience an increase in CAG repeat instability. In addition, CBP has been shown to regulate the *hedgehog* pathway.[255] Mutations in Sonic hedgehog have been identified in patients with holoprosencephaly,[256,257] a cerebral malformation causing severe mental retardation. It remains unclear if the cognitive defect is solely due to the developmental cerebral malformation.

Conclusion

We have reviewed several single gene human mental retardation disorders for which mutations in the Drosophila ortholog result in deficient learning and long-term memory. We have also shown that synaptic dysfunction is present in Drosophila mutants for genes involved in human mental retardation. This reinforces the notion that, although divergent from a neuroanatomic perspective, flies and humans have conserved molecular pathways for plasticity, memory and cognition. It also supports the idea of using Drosophila as a screening tool for studying genetic interactions, determining spatio-temporal requirements for a given gene and identifying pharmacological agents to treat intellectual disabilities. The next challenge will be to understand the genetic network in which a given gene is operating in vivo for cognitive processes and to treat intellectual disabilities using a multi-target approach that minimizes the risk of side effects or paradoxical responses.

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