Cell. Mol. Life Sci. 63 (2006) 975–988
1420-682X/06/090975-14
DOI 10.1007/s00018-006-6023-9

Cellular and Molecular Life Sciences

Dunces and da Vincis: The genetics of learning and memory in *Drosophila*

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Online First 5 April 2006

Abstract. Progress towards amelioration and eventual cure of human cognitive disorders requires understanding the molecular signaling mechanisms that normally govern learning and memory. The fly *Drosophila melanogaster* has been instrumental in the identification of molecules and signaling pathways essential for learning and memory, because genetic screens have produced mutants in these processes and the system facilitates integrated genetic, molecular, histological and behavioral analyses. We discuss the behavioral paradigms available to assess associative learning and memory in the fly, the contributions learning and memory mutants have made to our understanding of the molecular mechanisms that govern learning and memory, and predictions stemming from the nature of the affected genes. Furthermore, we consider the multiple well-established behavioral assays available and the powerful molecular genetics of the fly with regard to development of models of human cognitive disorders and their pharmacological treatment.

Keywords. Learning and memory, conditioning, *Drosophila*, neurogenetics, mutants.

Introduction

The ability to modify behavioral responses dependent on experience (learning) and retention of this change for variable lengths of time (memory) characterize all animal species. Learning and memory can be viewed from a geneticist's perspective as (complex) biological traits subject to genetic dissection. The genetic basis of behavioral traits such as geotaxis had been recognized, and analyses were initiated using *Drosophila melanogaster* as early as the 1960's [1, 2] because its hereditary mechanics were largely known and it was easy to propagate large numbers of genetically identical animals. Isolation of single-gene mutants in behavioral traits such as phototaxis and circadian rhythmicity [3–5] was followed by the demonstration that *Drosophila* can be conditioned, the proposal that single gene mutants perturbing normal learning and memory can be isolated [6] and the identification of the first such mutant *dunce* [7]. This initial breakthrough was followed by 3 decades of searching for genes involved in learning and memory capitalizing on the sophisticated *Drosophila* molecular and classical genetics (additional recent reviews [8–11]).

Modern behavioral analyses are facilitated by the development of sophisticated transgenic molecular tools, mediating temporal and spatial specific regulation of transgene expression. Recent development of transgenic systems that allow simultaneous tissue and temporal specific regulation of gene expression [12, 13] promise to advance our understanding of learning and memory far beyond gene discovery brought by neurogenetic analyses to date.

Learning and memory paradigms in *Drosophila*

Benzer's proposal that the biological processes governing learning and memory can be revealed by mutant analyses was followed by genetic screens aiming to identify mutants in the processes. Searching for learning mutants is unlike mutant screens aiming to isolate developmental or anatomical mutants which focus on visible aberrations. Failure to learn (or remember) is a phenotype inferred from the behavioral responses of the flies. Learning precipitates changes in the representations of stimuli in the

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nervous system. These changes are assessed by the animals' performance in tasks designed to evaluate these new learning-induced stimulus representations. However, performance is an indirect measure and not always an accurate estimate of an animal's actual learning and memory. Performance in the test task may not appear significantly changed immediately after behavioral training, indicating no learning-dependent behavioral change; but if memory of the same experience exists, it clearly indicates that learning actually occurred [14]. Nevertheless, provided they are not deficient in sensory modalities required to perceive and process the stimuli, animals unable to (sufficiently) modify their response to the conditioned stimuli are potential mutants. *Drosophila* are capable of learning in a variety of positively or negatively reinforced associative or non-associative tasks utilizing olfactory, visual and tactile stimuli, and remember what they are taught for a significant portion of their lives (reviewed in [15, 16]). There are two predominant behavioral paradigms used to assess associative learning and memory in *Drosophila*, olfactory and courtship conditioning. We will not discuss non-associative learning paradigms in this review, but they are summarized in [15].

Quinn, Harris and Benzer developed a negatively reinforced operant conditioning paradigm (QHB paradigm) [6] where flies learn to selectively avoid one of two odors they encounter which has been paired with electric footshock. A second, more robust, negatively reinforced, Pavlovian conditioning assay was established later [17, 18]. The odor (conditioned stimulus CS) is presented concurrently with multiple electric footshocks (unconditioned stimulus US), followed by another odor in the absence of the footshock reinforcer. Learning is significantly higher in this paradigm, and memory of the association is retained for at least 24 h. The training cycle can be repeated sequentially (massed), or with a rest period between cycles (spaced), with the latter resulting in memory of the association that can last 5–7 days [19]. The robustness and reliability of this assay has made this paradigm the method of choice in recent mutant screens and characterization of new learning and memory mutants. In addition, a positively reinforced variant paradigm utilizing sucrose to forge associations with particular odors was recently introduced [20].

Memory of the conditioned association in the negatively reinforced Pavlovian paradigm has been genetically and pharmacologically subdivided to phases that parallel those of other invertebrate and vertebrate models [10, 21–23]. The earliest possible time to reliably test the performance of the flies after conditioning is 2.5–3 min. Thus, it is impossible to directly assess learning/information acquisition in these paradigms, so the term 'immediate memory' denotes the earliest reliable performance measure after training [24, 25]. In addition to immediate memory, *Drosophila* exhibit short-term memory (STM), which decays in less than an hour and is independent of transcription and translation and three types of consolidated memory. Middle-term memory (MTM) is thought to last from 1 to 4 h and requires new protein synthesis from pre-existing messages. Anesthesia-resistant memory (ARM) is independent of protein synthesis, lasts around 24 h and is induced after a single or multiple massed training episodes. In contrast, bona fide long-term memory (LTM) is induced after multiple spaced training episodes and requires de novo transcription and translation [10, 19, 26, 27].

Conditioned courtship suppression is an operant conditioning paradigm [28, 29], based on the more ethologically relevant natural sexual behavior and engages visual, chemosensory and auditory information (reviewed in [28, 30]). Although mated females induce courtship, they block copulation attempts, and the rejected males tend not to court virgin females for 2–3 h, or fertilized females for 1 day [28, 31]. Extended exposure to mated females presumably leading to multiple spaced rejections leads to 9-day conditioned courtship suppression [32]. Though robust and requiring fewer animals, this paradigm is more tedious and has not been used as a mutant screening method of choice.

Drosophila can be conditioned to visual stimuli tethered to a wire and suspended while flying in the center of an arena with T-shaped patterns as landmarks. The fly is negatively conditioned when heading towards one of the patterns by heating, while its movements transduced via the tethering wire are measured by a torque meter [33, 34]. Memory is measured by testing for its orientation preference with respect to the landmarks. Although it is robust and has generated novel findings regarding visual learning and memory [35–37], the requirement for highly sophisticated equipment has been prohibitive for its use as a tool in mutant screens.

Another spatial operant paradigm requires the flies to prefer one side of a small chamber because crossing the imaginary midline results in punishment by heating the entire chamber [38]. Learning and memory of this spatial preference can last at least 2 h. Significantly, the 'heat-box' learning paradigm is completely automated, lending itself to large-scale mutant screens [39].

Genetic dissection of learning and memory

Two broad classes of genes can be expected to perturb learning and memory when mutated: first, genes essential for the development and maintenance of neuronal ensembles required for the formation, storage and retrieval of the conditioned behavioral response; second, genes implicated in the biochemistry of formation, storage and retrieval of the conditioned response within these neurons. Histological analyses often reveal mutations in the

first class of genes. Furthermore, if reversal of the behavioral defect (rescue) by expression of transgenically introduced normal genes requires transgene expression throughout development, it is suggestive of a gene of the first class. In contrast, if transgene expression prior to conditioning suffices for rescue, the gene likely belongs to the second class. With these considerations in mind, we review the results of screens to identify and characterize components of learning and memory processes in *Drosophila* (Table 1).

A number of mutants exhibiting reduced learning and memory were isolated in the pioneer chemical mutagenesis screen [6, 40], but attesting to the difficulty of cloning mutant genes harboring point mutations after chemical mutagenesis, some of the affected genes are still unidentified. Nearly parallel chemical mutagenesis screens (summarized in [15]) searching for structural defects in *Drosophila* brains provided evidence that perturbations of the Mushroom Bodies (MBs) and the Central Complex (CC) result in defective olfactory learning [41, 42]. The MBs are bilaterally symmetrical structures in the protocerebrum comprising approximately 2500 neurons per brain hemisphere essential for learning and memory in *Drosophila* and other insects [11, 43–45]. The observation that Dnc is preferentially distributed in the MBs [46], was followed by screens 'biased' to identify genes expressed in these neurons using 'enhancer trapping' transposons and yielded novel mutants [47]. Unbiased genetic screens utilized P-transposons to 'tag' mutated genes for quick identification and cloning, but also facile generation of additional alleles by imprecise excision of the element [48], yielded a number of additional learning and LTM mutants (Table 1).

The emerging DNA microarray technology was quickly enlisted to search for novel learning and memory genes using purely molecular methods. Based on the observation that the LTM form of consolidated memory requires de novo transcription, the screen was biased to identify genes transcribed after spaced Pavlovian olfactory conditioning [19]. That initial screen of only 1542 genes was reported to have identified 129 genes expressed differentially after LTM-inducing conditioning [10]. Although the identity of most of these genes is still unknown, a number of them were also identified as important for memory in 'unbiased' genetic/behavioral screens [10]. This validates both approaches and suggests that additional rounds of such molecular screens, specifically honed to address particular forms of memory, will likely reveal novel genes and molecular pathways important for these processes.

Identification of molecules important for learning and memory in genetic and molecular screens provided the necessary background to engage additional genes based on 'best-candidate' approaches capitalizing on the increased sophistication of transgenic techniques [12, 13]. These 'reverse-genetic' methods have been augmented with RNA interference (RNAi) techniques coupled to the UAS/GAL4 strategies to provide a novel tool to silence genes in a tissue-restricted manner [13]. This method has been shown effective in phenocopying non-associative [49] and associative behavioral phenotypes [50] and holds much promise for the future.

cAMP signaling cascade mutant

This signaling cascade is perhaps the most extensively characterized in all models of learning and memory, partly because two of the mutants identified in the original chemical mutagenesis screen, *dunce* (*dnc*) and *rutabaga* (*rut*), affect genes encoding a cyclic AMP (cAMP) phosphodiesterase II [7, 51, 52] and a Ca^{2+}/calmodulin-sensitive adenylyl cyclase [53, 54], respectively. Both genes are preferentially expressed in the MBs [46, 55], and their mutants exhibit immediate memory deficits in both the QHB and the Pavlovian olfactory paradigms [40] and courtship conditioning [31, 56, 57]. Furthermore, mutants in both genes exhibit deficits in habituation and sensitization [58–62], clearly indicating that cAMP signaling is central to multiple types of associative and non-associative learning and memory in *Drosophila*. The prediction that the Rut adenylyl cyclase is activated via a G-protein-coupled receptor was tested by transgenic expression of a constitutively active form of a $G\alpha_s$ subunit in MB neurons, which precipitated severe deficits in the Pavlovian assay [63]. Since similar expression of a regulated wild-type $G\alpha_s$ protein did not perturb learning, regulation of G_s signaling and cAMP levels are essential for normal learning and memory in *Drosophila*.

An additional molecule implicated in cAMP signaling identified in the Benzer mutant screen is the pituitary adenylyl cyclase-activating peptide (PACAP)-like protein. This peptide is encoded by the gene *amnesiac*, and its function is essential for the anesthesia-sensitive MTM component of consolidated memory [64, 65]. Conceptually, then, the Amn peptide could signal through a $G\alpha_s$-coupled receptor to Rut to increase cAMP levels in neurons, which could be further modulated by the activity of Dnc. This model predicts that Amn should accumulate in neurons other than those expressing Rut, and this was verified with the demonstration that two neurons in the adult brain (the Dorsal Paired Medial neurons, DPMs) express *amn*. A single neurite from each DPM elaborates an extensive projection network on each of the MB lobes [64], sites of preferential Rut accumulation [55, 66]. Therefore, Amn-dependent modulation of Rut activity after training could prolong the elevation of cAMP in the MBs, a condition necessary for MTM formation [21]. Consistent with this, DPM neuronal output was required during the consolidation phase and not during acquisition or recall of olfactory memories in the Pavlovian paradigm

Table 1. Summary of screens to identify molecules essential for *Drosophila* learning and memory.

Mutant	Product	Biochemical pathway	Expression pattern	Conditioning paradigm	Behavioral deficit
Forward EMS screens					
dunce (dnc)	cAMP phosphodiesterase	cAMP	MB	P.O.C./O.O.P./C.C.	learning
rutabaga (rut)	calcium/calmodulin-activated adenyl cyclase	cAMP	MB	P.O.C./O.O.P./C.C.	STM
amnesiac (amn)	neuropeptide(s) related to PACAP	cAMP	DPM	P.O.C./O.O.P./C.C.	STM
turnip (tur)	–	PKC?	–	P.O.C./O.O.P./C.C.	non-associative
radish (rsh)	–	?	?	P.O.C./O.O.P./C.C.	ARM
ala	α-lobes absent	MB development	–	P.O.C.	LTM
P-element screens					
linotte (lio)	receptor tyrosine kinase *derailed*	MB development	MB and CX	P.O.C.	learning
latheo (lat)	component of the origin recognition complex	MB development	MB and NMJ (larval)	P.O.C.	learning
ignorant (ign)	ribosomal S6 kinase (S6KII)	ERK/MAPK	–	O.O.P/H.B.C	learning
P-element screens for LTM mutants					
nalyot (nal)	Myb-related *Adf1* transcription factor	–	CNS	P.O.C.	LTM
crammer (cre)	cysteine protease inhibitor	–	MB and glia	P.O.C.	LTM
milord (pum)	ribonucleoprotein translocation/translation repression	RNA transport	MB	P.O.C.	LTM
staufen (stau)	ribonucleoprotein translocation	RNA transport	–	P.O.C.	LTM
norka (osk)	ribonucleoprotein translocation	RNA transport	MB	P.O.C.	LTM
krasavietz (eIf-5C)	translation initiation factor	RNA transport	MB	P.O.C.	LTM
murashka (mur)	–	–	'murashka' neurons	P.O.C.	LTM
Enhancer detector P-element screens					
DCO	catalytic subunit of PKA	cAMP	MB	P.O.C.	STM
leonardo (leo)	14-3-3	Ras/Raf/MAPK?	MB	P.O.C.	learning
volado (Vol)	α-integrin	cell adhesion	MB	P.O.C.	STM
fasciclinII (fasII)	fasciclin II	cell adhesion	MB	P.O.C.	STM
Microarray screens					
staufen (stau)	ribonucleoprotein translocation	RNA transport	–	P.O.C.	LTM
pumullio (pum)	ribonucleoprotein translocation/translation repression	RNA transport	MB	P.O.C.	LTM
oskar (osk)	ribonucleoprotein translocation	RNA transport	MB	P.O.C.	LTM
eIF-5C	translation initiation factor	RNA transport	MB	P.O.C.	LTM
Best-candidate approach					
CREB	cAMP-response element binding protein	cAMP	–	P.O.C.	LTM
CamKII	Cam Kinase II	CamKII	CNS	C.C	learning
Notch (N)	Notch	Notch	–	P.O.C./C.C	LTM
PKA-RI	regulatory subunit of PKA	cAMP	MB	P.O.C./C.C	STM
Gas	guanosine-triphosphate binding protein α subunit	cAMP	–	P.O.C.	learning
PKC	protein kinase K	PKC	–	C.C	learning
αPKM	atypical PKM	PKC	–	C.C	ARM
synapsins (syn)	synapsins	cAMP?	CNS	P.O.C./C.C/H.B.C	learning
TH/dopamine	dopamine receptors	cAMP?	CNS	P.O.C.	learning
TβH/octapamine	octapamine receptors	cAMP?	CNS	P.O.C.	learning
dNR1	NMDA receptors	–	multiple CNS neurons	P.O.C.	learning

P.O.C., Pavlovian Olfactory Conditioning; O.O.P., Operant Olfactory Conditioning; C.C., Courtship Conditioning; H.B.C., Heat Box Conditioning; CNS, Central Nervous System; MB, Mushroom Bodies; CX, Central Complex; DPM, Dorsal Paired Medial neurons; NMJ, Neuromuscular Junction; STM, Short-Term Memory; LTM, Long-Term Memory; ARM, Anesthesia-Resistant Memory; 'murashka' neurons, neurons sending projections into mushroom bodies and lateral horn.

[65]. However, it is still unclear how and when the DPMs become activated by pairing of the odor/footshock stimuli such that Amn peptide is released onto the MB lobes to modulate Rut activity.

The requirement for cAMP modulation in olfactory learning and memory and the preferential distribution of Dnc and Rut in the MBs predicted involvement of the cAMP-activated protein kinase (PKA) in these processes. This was tested initially using the 'candidate gene' approach. Ubiquitous expression of transgenes encoding a vertebrate PKA peptide inhibitor, as well as fragments of the vertebrate regulatory subunit II (RII), disrupted immediate memory in the Pavlovian olfactory conditioning assay [67]. Furthermore, a mutant in the catalytic subunit of PKA (*DC0*) was identified in the screen for MB genes [68], enabling a direct test of the hypothesis. The DC0 protein along with the RI and RII regulatory subunits of the PKA holoenzyme [66, 69] accumulate preferentially in MBs. Consistent with this, reduction of cAMP-inducible PKA activity in *DC0* mutants resulted in immediate memory scores indistinguishable from those of *rut* mutants [70] and nearly eliminated MTM [68, 71]. Based on these results, using site-selected P-element mutagenesis, mutations in the gene for the RI subunit were isolated, and mutants were shown to be defective in olfactory immediate memory and MTM [69] as well. In addition, they appeared to disrupt memory, but not learning in the courtship conditioning assay [57]. Therefore, the Rut-produced cAMP likely activates PKA, which is essential for learning and memory.

Because cAMP signaling had been implicated in LTM in other experimental systems [72, 73], and the transcription factor CREB is a major PKA phosphorylation target, reverse genetic methods were employed to generate a strain harboring an inducible dominant negative CREB transgene (*dCREB2b*). Expression of this dominant negative CREB specifically abolished LTM induced by spaced training in the Pavlovian paradigm, while immediate memory and the ARM form of consolidated memory were unaffected [74]. Thus, de novo transcription mediated by CREB proteins appears essential for LTM in *Drosophila*, as for other species. However, although necessary, dCREB2 alone does not seem sufficient for LTM formation. The original finding of Yin et al. [75] that transgenic overexpression of an activated form of CREB was sufficient to induce LTM has not been reproduced and is in fact disputed by a recent report [76]. Nevertheless, the cumulative data clearly indicate that cAMP signaling is essential for *Drosophila* associative and non-associative learning and memory. The importance of this signaling pathway for olfactory learning and memory may be reflected in the relative ease that multiple members of the cascade were isolated by mutagenesis screens, an additional factor being that all these original mutations were homozygous viable. However, even total loss of Rut cyclase activity and near lack of DC0 reduce immediate memory and MTM by about 50% [70], suggesting that additional molecules and signaling pathways contribute to normal olfactory learning and memory. Isolation of additional learning and memory mutants, unlikely to participate in cAMP signaling, further supports this notion.

Other major signaling cascade mutants

Calcium/calmodulin-dependent protein kinase II (CamKII) is a major pre-synaptically enriched neuronal kinase which can adopt different activity states depending on its autophosphorylation [77]. The contribution of this kinase

Table 2. Human cognitive disorders modeled in *Drosophila*.

Human disorder	Protein	*Drosophila* model	Behavioral assay	Behavioral deficit
Tauopathies	Tau	transgenic human and *Drosophila* wild-type *tau*	olfactory conditioning	learning, middle-term memory
		transgenic human wild-type and mutant *tau*	locomotion	
Alzheimer's	Aβ40/Aβ42	transgenic human *Aβ40/Aβ42*	olfactory conditioning	learning
	App	*Drosophila Appl* mutant	phototaxis/chemotaxis	
Down's syndrome	Dscr1	*Drosophila nebula* mutant	olfactory conditioning	learning, long-term memory
		transgenic *Drosophila nebula*	olfactory conditioning	learning, long-term memory
Fragile X	Dfmr1	*Drosophila Dfmr1* mutant	courtship conditioning	learning
Neurofibromatosis	neurofibromin (Nf1)	*Drosophila Nf1* mutant	olfactory conditioning	learning

in *Drosophila* learning and memory was explored using 'reverse genetics'. Global transgenic expression of a CamKII peptide inhibitor is detrimental to learning and memory in the courtship conditioning paradigm [78]. Activity of the kinase is required in the MBs and parts of the CC for normal memory formation in the absence of visual cues driving the behavior. In the presence of visual cues, courtship is not suppressed immediately after training, but memory of the conditioning is normal [29, 79], a case where (immediate) performance does not reflect learning. Moreover, expression of a constitutively active kinase in the olfactory information-processing antennal lobes and extrinsic MB neurons enhances learning [80]. These findings suggest that in this conditioning paradigm, which engages multiple anatomical sites, CamKII may play distinct biochemical roles for learning and memory. However, the normal distribution of the protein in the brain and whether similar effects on neuroplasticity may be revealed in a different conditioning paradigm are currently unknown.

Another kinase whose contribution to *Drosophila* learning and memory has been explored is the diacylglycerol and Ca^{2+}-dependent protein kinase C (PKC), initiated by the isolation of the *turnip* mutant in the Benzer screen. However, although *turnip* mutant flies exhibit reduced PKC activity, the mutation does not map near any of the PKC loci [81], and the affected gene has not been identified yet. Furthermore, it appears that the learning deficiency of *tur* mutants may be a consequence of their reduced locomotion and poor responses to the electric footshock US used in the QHB and Pavlovian olfactory assays rather than defective associative abilities [82]. In contrast, *tur* mutants exhibit normal learning but defective memory in courtship conditioning [83], suggesting that PKC activity may actually contribute to *Drosophila* behavioral plasticity. This hypothesis was tested by temporally restricted expression of a selective PKC inhibitor peptide in the brain. These transgenic flies exhibit normal memory in the courtship conditioning assay, but fail to suppress courtship after training, indicating that they dissociate learning from performing the task requisite to assess it [14]. It is unknown whether inhibition of PKC activity affects learning or memory in other conditioning paradigms, and in the absence of bona fide mutants, it is difficult to determine whether typical PKC molecules play a role in *Drosophila* learning and memory. In contrast, the atypical, diacylglycerol and Ca^{2+}-independent aPKCζ, important for vertebrate physiological neuroplasticity [84, 85], appears important for *Drosophila* behavioral plasticity. Transgenic expression of a murine or *Drosophila* constitutively active form (PKM) within a narrow post-training window in the Pavlovian paradigm enhanced ARM, but appeared not to affect LTM. This effect was eliminated either by a dominant negative form of the enzyme or pharmacologically by chelerythrine [86], a modestly selective PKM inhibitor. Though these results are highly provocative, it is difficult to assign an unequivocal role for PKM in normal *Drosophila* memory without genetic mutants, or temporal and/or tissue restricted silencing of the endogenous gene.

Mutants in cell surface molecules

Two cell adhesion molecules were identified in the screen for genes preferentially expressed in the MBs. *Volado* encodes an α-integrin, in whose absence immediate memory and STM elicited by Pavlovian olfactory conditioning are disrupted [87]. The importance of this single trans-membrane domain protein in neuronal function was also demonstrated by deficits in synaptic plasticity in mutant larval neuromuscular junctions (NMJs) [88], but the structure of the MBs and the NMJs appear normal in *vol* mutants. Integrins form heterodimers, and the dimerization partner of Vol is currently unknown. Moreover, the learning and memory-relevant signaling mechanisms engaged by this molecule are currently unknown, but one attractive possibility is engagement of the Ras/Raf/MAPK cascade, as has been reported for integrin function in other types of cell-to-cell interactions [89]. The second surface molecule encoded by the *fasciclinII* gene is a *Drosophila* homolog of the vertebrate and invertebrate cell adhesion molecules (CAMs), and *fasII* mutants are deficient in formation of immediate memory and STM in the Pavlovian olfactory assay [90]. Similar to its *Aplysia* homolog (ApCAM), FasII is involved in synaptic plasticity on the pre-synaptic side of the NMJ [91, 92]. Consistent with ApCAM, rapid reduction of FasII at the NMJ (possibly through internalization) coincides with MAPK activation [93], suggesting that a similar signaling mechanism may be operant in the MBs as well.

A surprising molecule implicated specifically in LTM formation is the transmembrane receptor Notch. This molecule has been well studied in multiple organisms for its role in cell-type specification in various developmental contexts. Its normal role in mature neurons has not been examined [94], though it may play a role in dementia associated with Alzheimer's disease. Similar to processing of Amyloid Precursor Protein, ligand-bound N receptor is cleaved by a gamma-secretase-like protein, and alterations in N signaling have been associated with the Alagille and Casadil syndrome dementias [95–97]. Significantly, RNAi-mediated silencing of *N* within the MBs specifically inhibited LTM in the Pavlovian olfactory paradigm, while ubiquitous reduction of the receptor using a temperature-sensitive allele resulted in LTM deficits after courtship conditioning [98]. Similarly, a dominant negative N specifically inhibited LTM but not ARM, while overexpression of a normal receptor yielded enhanced LTM even after a single cycle of olfactory conditioning. Important questions such as the nature of N lig-

ands in the brain, the neurons where the receptor accumulates in and the intracellular mechanisms engaged by N activation to mediate protein synthesis-dependent LTM currently remain unanswered.

Neurotransmitter and synaptic function mutants

Dopa decarboxylase (Ddc) is essential for the biosynthesis of dopamine and serotonin. An early report indicated that temperature-sensitive *Ddc* mutants harboring reduced serotonin and dopamine are learning deficient in the QHB and the courtship conditioning paradigms [99], but this work has not been verified [9]. However, Tyrosine Hydroxylase (TH) is also required for dopamine production, and inhibiting neurotransmission from TH-positive neurons nearly abolished immediate memory elicited by negatively, but not positively reinforced Pavlovian conditioning [20]. Moreover, mutants in the tyramine β-hydroxylase (TβH) encoding gene are unable to synthesize the *Drosophila* analog of epinephrine, octopamine [100]. Immediate memory of positively, but not negatively reinforced Pavlovian conditioning is disrupted in these mutants [20], indicating that both neurotransmitters are essential for *Drosophila* olfactory learning and memory and engage MB neurons, but apparently are used differentially to mediate the positive or negative US in Pavlovian conditioning [20].

Interestingly, MB neurons themselves utilize taurine, aspartate and glutamate [101], and glutamatergic neurotransmission appears important for behavioral neuroplasticity. Transposon insertions in the *dNR1* NMDA receptor gene result in impaired immediate memory in Pavlovian conditioning, but the deficit is overcome with intensive training. However, even after intensive spaced training LTM is disrupted, clearly indicating the importance of the dNR1 receptor [50]. *Drosophila* contains a second receptor gene, *dNR2*, whose contribution to learning and memory is unknown since mutants are not available. Interestingly, both proteins are distributed throughout the brain, but most prominently in particular cells that surround the dendrites of MB neurons, potentially modulating stimuli reaching the latter [50].

Although highly regulated and complex, neurotransmitter release depends on the function of the synaptic vesicle-associated protein synapsin [102]. Targeted P-element mutagenesis yielded surprisingly viable mutations in the single *Drosophila* synapsin gene. As suspected, these mutants were defective in a number of complex behaviors, including learning in Pavlovian, courtship and heat box conditioning paradigms [103].

RNA transport and translation mutants

Mutants in these processes were identified in an unbiased P-transposon screen and independently identified in a microarray-based screen for genes transcriptionally altered by LTM-producing spaced Pavlovian conditioning [104]. These genes fit two broad classes: The first class encodes proteins involved in translational control, such as the translational repressor Pumillio, also identified by its LTM-deficient mutant alleles *milord*^{1} and *milord*^{2}, the localized messenger RNA (mRNA) regulator CPEB/Orb, and the translation initiation factors eIF-2G and eIF-5c, the latter also identified as the LTM mutant *krasavietz* [104]. The second class contains genes involved in intracellular mRNA transport and includes *staufen* (*stau*), and proteins involved in *stau* localization such as Moesin, and Oskar, which was also identified by its allele *norka* in the LTM mutant screen. Therefore, it appears that as in *Aplysia*, cultured neurons [105], memory formation and storage in *Drosophila* require transport and local translation of pre-existing and de novo transcribed RNAs. Additional results from these screens will likely enrich the collection of molecules and mechanisms requisite for LTM. It is noteworthy that all of these novel LTM genes whose pattern was examined are preferentially expressed in the MBs [104].

Other mutants

Consolidated memory mutants

Mutants of the *radish* (*rsh*) gene isolated in a Benzer screen exhibit near normal learning, but are specifically impaired in the ARM component of consolidated memory [106]. The nature of the affected gene remains controversial. One group claims to have identified the affected gene as Phospholipase A2, but have not identified within this gene the lesion likely causal of the original *rsh*^{1} mutation and have not rescued the memory deficit of the original *rsh*^{1} mutant with overexpression of the PL-A2 gene [107]. Unpublished results from a different group suggest that the *rsh*^{1} mutation is located in a different neighboring novel gene. Unless overexpression of this gene improves ARM non-specifically, it was reported to revert the ARM deficiency of the original *rsh*^{1} mutant [E. Folkers, and W. Quinn, 45th *Drosophila* Research Conference]. Moreover, the expression pattern of the two genes is very different. PL-A2 accumulates in a complex neuronal network with some of the neurons apparently engaging the MB dendritic region [107], while the alternate novel protein preferentially accumulates in the lobes of the MBs. Interestingly, ARM is nearly abolished if neurotransmission from the MBs is blocked [27]. This information suggests that the novel gene mutated in *rsh*^{1} flies is appropriately expressed to be the correct candidate. Since this is the only known mutant to specifically disrupt ARM, it is essential to unequivocally establish which of the two genes (or both?) is responsible for the *rsh* phenotype.

The mutant *nalyot* (*nal*), which was isolated in the unbiased P-element-mediated behavioral screen for memory-deficient strains, disrupts expression of the Myb-related transcription factor Adf1 expressed ubiquitously in the brain. Immediate memory in the Pavlovian conditioning task is reduced, but 24-h and 7-day LTM are nearly absent [108]. Given the dependence of LTM on transcription, this is an expected phenotype for mutants in a transcription factor. However, regulation of Adf1 expression is surprisingly tightly regulated, because accumulation of transgenic protein during development appears to affect memory adversely, or precipitate lethality even if confined to the nervous system. Consistent with a role in nervous system development, *nal* mutations affect the presynaptic structure of larval NMJ but do not appear to affect synaptic transmission [108]. The transcriptional targets of *nal* are currently unknown.

A novel mutant, *crammer* (*cer*), identified in a different unbiased screen for LTM mutants, reduces expression of a gene encoding an apparent inhibitor of the cysteine proteases known as cathepsins. The protein is preferentially distributed in adult MBs and surprisingly in glia, as well [109]. Interestingly, overexpression of a *cer* transgene in glia, but not in the MBs, disrupted LTM in wild-type flies. Although it is presently unclear whether overaccumulation of this inhibitory protein affects glia developmentally, it opens up the possibility that normal LTM may require these non-neuronal cells. Furthermore, the nature of the affected gene suggests that regulation of cathepsin protease activity is essential for LTM, and similar unexpected findings will likely be revealed by characterization of additional mutants.

Learning and memory mutants

Mutations in *leonardo* (*leo*) were identified in the MB enhancer trap screen. Mutations that diminish accumulation of the protein in the MBs result in immediate memory deficits and proportionally compromise MTM and later consolidated memory [24, 110]. The gene encodes two ζ isoforms of the 14-3-3 family, proteins highly enriched in all metazoan nervous systems and conserved in all eukaryotes. The protein is important for regulation of Raf kinase activity in developmental contexts [111], but this and other members of the protein family have been implicated in the regulation of many different signaling cascades and processes [112], making an unequivocal assignment of the role of this protein difficult. One possible role is suggested by depleting the protein from the NMJ, where it normally accumulates. This results in deficient synaptic transmission and physiological neuroplasticity [113], possibly because of its documented interaction with the *Slowpoke* Ca^{2+}-activated potassium channel [114]. However, it is unclear whether such a mechanism, or misregulation of Raf activity and MAPK signaling is responsible for compromised MB-dependent olfactory learning and memory.

An unbiased screen to identify mutants that disrupt operant conditioning in the heat box yielded the mutation *ignorant* [115], which was found to disrupt the gene encoding Ribosomal S6 Kinase II (RS6KII). Although essential for many vital cellular functions, RS6KII is also associated with physiological and behavioral neuroplasticity [116] by modulation of ERK/MAPK signaling. Interestingly, flies lacking the gene are normal in operant conditioning, but defective in Pavlovian learning. Conversely, *ignorant* mutants missing only the 5′ portion of the gene are dominantly deficient for immediate memory in either heat box or Pavlovian conditioning, suggesting complex regulation of the gene or protein. Neurons where RS6KII accumulates are currently unknown.

Developmental mutants

Latheo (*lat*) mutants isolated in a P-transposon screen exhibit deficient immediate olfactory memory, MTM and consolidated memories [117], likely due to reduction in MB size. This is consistent with encoded protein, a factor essential for recognition of the origin of DNA replication [118]. Lat protein is also present at the larval NMJ, and *lat* mutants are defective in synaptic plasticity [119], suggesting that the protein may have multiple functions. Another mutant identified in the same behavioral screen, *linotte* (*lio*) [120], disrupts the de*railed* gene encoding a receptor tyrosine kinase, essential for development of the central brain, including the MBs [121, 122], and disrupts immediate memory and STM in the Pavlovian assay [120]. In both of these cases alterations in the normal structure of the MBs resulted in compromised behavioral neuroplasticity as previously suggested by mutant screens [42]. In addition, recent analysis of the low-penetrance mutation *alpha-lobes-absent* (*ala)*, which results in elimination of the α and α' or the β and β' MB collaterals, suggested that the α/α' lobes are specifically important in formation or retrieval of LTM [123].

Drosophila models of human learning and memory disorders

One of the outcomes of sequencing the genomes of humans and model organisms was the high degree of similarity in the proteins they encode, and clearly *Drosophila* shares molecules that govern learning and memory with humans. For example, in humans, the mental retardation condition Rubinstein-Taybi syndrome results from disruption of CREB-dependent transcription [124], similar to the loss of LTM in *Drosophila* upon inhibition of CREB activity. Moreover, *rsk2* mutations cause the Coffin-Lowry mental retardation syndrome [125], reminiscent of the *Drosophila* mutant *ign*. Such similarities in cognitive disorders support the use of *Drosophila* in exploring the ge-

netics of many human nervous system diseases. Neurodegenerative disorders have also been modeled in *Drosophila*, but will not be discussed here. *Drosophila* models contribute to understanding mechanisms of learning and memory, but also provide a versatile system to test pharmaceuticals designed to stop disease progression or ameliorate its symptoms.

Neurofibromatosis

Mutations in the neurofibromatosis 1 (*Nf1*) gene produce a dominant disorder in humans characterized by benign neuronal tumors and dysfunction manifested as movement and learning disorders [126]. The protein is a Ras-inactivating, GTPase activating protein (GAP), suggesting that Ras/Raf signaling is important for learning and memory in humans. *Drosophila* lacking the fly homolog of Nf1 are severely impaired in immediate memory and all subsequent forms of consolidated memory [127]. Similarly, elimination of Nf1 activity in transgenic mice resulted in severe learning deficits, reversible either by pharmacological inhibition of N-Ras and K-Ras, or by combining the *Nf1* mutants with *N-ras* or *K-ras* mutant heterozygotes [128]. The *Drosophila* and mouse data strongly suggest that the learning and memory effects are caused by misregulation of Ras activity. Surprisingly, in *Drosophila*, the immediate memory deficits were rescued by overexpression of a constitutively active PKA catalytic subunit transgene [127], and Nf1 was shown to regulate a component of G-protein-activated adenylyl cyclase activity in fly and in mouse brains [129]. This suggests that the protein may have a dual function, suppressor of Ras activity and enhancer of cAMP signaling. However, whether Nf1 regulates Ras activity with respect to *Drosophila* learning and memory remains unknown. These observations suggest either that cAMP-mediated suppression of Ras activity is required for normal learning and memory, or that the two systems may be complementary in mediating these processes. Particular stimuli may activate the adenylyl cyclase/cAMP cascade and suppress Ras signaling, while others engage a Ras, but not a cAMP mediated path. Learning and memory induced by distinct stimuli or conditioning paradigms may engage distinct signaling systems with the same ultimate goal, learning and memory formation. In addition, such signaling systems may be at least partially complementary, such that a compromise in one is partially complemented by hyperactivation of the other.

Tauopathies

Mutations that perturb the isoform composition or the coding region of the neuronal specific microtubule-binding protein Tau are associated with a number of dementias and neurodegenerative diseases, most notably Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) and Alzheimer's [130]. Transgenic expression of human *tau* leads to degeneration which is more pronounced when transgenes bearing FTDP-17-linked mutations are used [131]. To explore the role of TAU in the manifestation of the cognitive symptoms that accompany all Tauopathies, transgenic overaccumulation of vertebrate and *Drosophila* Tau was directed specifically to the MBs. This condition precipitated large deficits in learning and memory after Pavlovian conditioning, without neurodegeneration, suggesting that behavioural deficits precede the latter [132]. Accumulation of vertebrate Tau in larval motor neurons appears to block anterograde transport [133, 134], suggesting that the learning and memory deficits are precipitated by a similar blockade of synaptic vesicles in MB neurons. These conditions are thought to be akin to the situation in neurons of FTDP-17 and Alzheimer's patients prior to visible degeneration and neurofibrillary tangle (NFT) formation, and potentially underlie at least a part of their cognitive symptoms.

Amyloid-Aβ40/Aβ42

Accumulation of amyloid-β (Aβ) 40 and Aβ42 peptides in senile plaques observed in Alzheimer's patients has been suggested to be a primary event in disease pathogenesis and progression even though aberrant Tau accumulation is almost always an accompanying feature of the disease. To determine whether accumulation of these peptides alone is sufficient to cause behavioral and neurodegenerative pathology, Aβ40 and Aβ42 encoding transgenes were expressed in *Drosophila* nervous system from early development to adulthood. Age-dependent immediate memory deficits were observed in the Pavlovian olfactory assay in animals expressing either transgene. However, only Aβ42 accumulation led to amyloid-like deposits and degeneration, indicating that the two peptides have distinct roles in neurodegenerative processes, but accumulation of either is sufficient to cause learning impairments.

Down's syndrome critical region 1 gene/Nebula

Patients with complete or partial trisomy 21 exhibit the phenotypic abnormalities associated with Down's syndrome (DS), the most prominent of which is mental retardation. One of the genes included in the minimal region from chromosome 21, which causes the full DS phenotypes, normally expressed in the brain and overexpressed in the brain of DS fetuses is *DSCR1*. The gene encodes calcipresin, an inhibitor of the serine/threonine protein phosphatase calcineurin, which is essential for normal learning and memory in mice [135–137]. The hypothesis that overexpression of the *DSCR1* gene con-

tributed to the mental retardation phenotype of Down's patients was investigated with a *Drosophila* model. Loss-of-function mutants and transgenic animals overexpressing the *Drosophila* ortholog *nebula* (*nla*) exhibited severe immediate memory and consolidated memory defects after Pavlovian conditioning, without obvious developmental defects. Similar to DS fetal brains, *nla* mutants exhibited elevated calcineurin activity, indicating that in trisomy 21 humans, elevation of DSCR1 and increased calcineurin-mediated signaling likely contributes to the observed mental retardation [138]. Significantly, PKA activity and phosphorylated CREB were decreased nearly 50% as a consequence of increased calcineurin activity, adding another molecule to the cAMP-signaling cascade mediating learning and memory in *Drosophila*.

Fragile X syndrome/dfmr1

An elegant demonstration of the power of *Drosophila* in pharmacological amelioration of mental retardation-related symptoms was published recently and regards a common heritable mental retardation disorder, Fragile X syndrome, caused by loss of function of the *FMR1* gene. Loss of the *Drosophila* homolog *dfmr1* results in defective MB development, synaptic structure and function and memory in the courtship conditioning paradigm [139, 140]. To test the proposal that the cognitive defects in Fragile X patients result from enhanced metabotropic Glutamate Receptor (mGluR) signaling [141], fly *dfmr1* mutants were treated with mGlutR antagonists and lithium. Acute administration of the drugs to adult mutants resulted in restoration of both learning and memory deficits, while feeding the drugs to larvae eliminated the MB developmental anomalies in addition to restoration of behavioral plasticity [140]. These results suggest that similar treatment of human patients may ameliorate some of their cognitive symptoms. This adds another member to the collection of molecules involved in mRNA transport and regulation of local protein synthesis, processes that require the FMR protein [142], which are essential for learning and LTM

Perspectives

Research on olfactory learning and memory in *Drosophila* has identified a number of essential genes and molecular pathways. Homologs of genes discovered in *Drosophila* such as *rut*, *creb* and *vol* are also essential for mammalian learning and memory (reviewed in [11]), a demonstration of the conservation of molecular mechanisms that govern these processes across species. Although the collection of molecules and the pathways required for learning and memory has grown considerably, we are far from putting all the pieces of the puzzle together. Additional mutants are needed to elucidate mechanisms and to place currently disparate molecules in biologically meaningful pathways and processes. In addition, new experimental avenues have been opened recently by new tool development, as illustrated below.

Systems neuroscience of learning and memory

Development of transgenic systems that allow monitoring of neuronal activity, coupled with tissue-specific Gal4 drivers enabled direct visualization of the activity of particular neurons or entire neuronal assemblies in real time [143]. Such methods have been used to probe activity in the MBs [144, 145] and in antennal lobe neuronal assemblies in response to odors alone, or paired with electric shock delivered to a restrained fly [146]. Using a simplified single odor conditioning paradigm, Yu et al. show very convincingly odor-specific activation of specific neuronal assemblies within the antennal lobe. These activity signatures change specifically when odor is paired with electric shock, as different sets of neurons are recruited to respond to each shock-paired odor. Thus, odor representation in neurons of the antennal lobe changes contingent on pairing with the electric shock US. This short-lived memory trace, possibly representing short-term or working memory has also been described in bees [147], and its relationship to MB-dependent learning and memory in normal and mutant flies needs to be explored.

Learning and memory systems in the fly brain

How many different signaling systems mediate learning and memory in the fly? The cAMP system has been well established for olfactory learning and memory, but it is unlikely the only one. It remains a challenge to elucidate how other extant mutants fit into signaling pathways and which ones, and how they interact with the established cAMP pathway. Addressing these questions will require facile generation of double (and in some cases triple) mutants. Are signaling pathways unique to different types of learning and memory, such as operant, courtship, visual etc., or do the extant and future mutants affect all of them? The finding that negatively and positively reinforced Pavlovian olfactory conditioning employs different neurotransmitters argues that similar molecular distinctions may be uncovered for different types of conditioning paradigms. Are multiple areas in the brain engaged in learning and memory? Clearly the MBs are of cardinal importance for olfactory learning and memory, but in addition to the DPMs and antennal lobes, other neuronal networks [10, 50, 107] may be emerging as contributors to the processes. Courtship conditioning requires the MBs, CC and the antennal lobes, but also areas of the lateral protocerebrum [148], suggesting some de-

gree of specialization. Moreover, simple visual learning does not appear to require the MBs, but expressing the learned information in different contexts does [37]. Similarly, the neurons essential for heat box learning are not yet known, but memory in this paradigm clearly does not require the MBs [39]. *Drosophila* will be instrumental in addressing these fundamental issues in behavioral neuroscience because of the sophistication developed over the last couple of decades, the modern tools that allow a systems approach, but also importantly with more screens for additional mutants in associative and non-associative learning and memory.

Acknowledgements. The authors are indebted to Drs. Summer F. Acevedo, Elias Pavlopoulos and Anke R. Friedrich in particular and all members of the lab for numerous fruitful discussions. Work in the lab has been supported by grants from the NIMH and NSF (USA), the Marie Curie IRG (EU) and PENED (Greece) programmes.

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