I. INTRODUCTION

The power of genetics has been clearly demonstrated in studies of embryonic development. Systematic searches for mutant phenotypes during embryogenesis in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980; Johnston and Nusslein-Volhard, 1992; Wieschaus, 1996) and zebrafish (Grunwald and Eisen, 2002) have uncovered most of the genes controlling embryonic pattern formation. The number of genes involved is limited, and they can be categorized into specific functional groups according to their phenotypic effects. Many of the corresponding genes have been characterized molecularly, thereby identifying components of gene regulation and cell signaling pathways. Genes first discovered in flies have subsequently been identified in vertebrates, and basic genetic mechanisms controlling early development of invertebrates have been shown to be conserved in vertebrate systems. Thus, a driving force behind analysis of development has been gene discovery in flies, which in turn has provided experimental and conceptual tools to dissect mechanisms of morphogenesis in both invertebrate and vertebrate species.
For centuries it has been accepted that heredity contributes to learning and memory. The experimental evidence came in the mid-twentieth century when Tryon (Tryon, 1940) used bidirectional selection experiments to breed “bright” and “dull” strains of rats. Bright and dull rats were selected based on their abilities to learn to navigate a maze for a food reward and were mated among themselves. After several generations, rats from the “maze-bright” strain learned quickly, whereas those from the “maze-dull” strain learned much more slowly. Similarly in the blowfly *Phormia regina*, Hirsch and coworkers (T.R. McGuire and Hirsch, 1977; Zawistowski and Hirsch, 1984) bred flies showing high or low performance during classical conditioning of the proboscis extension reflex (PER). They were able to generate bright and dull strains of flies that had significantly different learning scores for classical conditioning.

These experiments established genetic components in learning and memory. The application of genetics in studying learning and memory started when Seymour Benzer at the California Institute of Technology brought to the field of *Drosophila* behavioral genetics the single-gene mutant approach in the early 1970s. He suggested that most genes involved with a complex trait might be identified by direct chemical mutagenesis to isolate mutations one gene at a time (Tully, 1996) — in much the same way that genetic screens were being used to study simpler processes. He and colleagues carried out genetic screening for learning and memory mutants in *Drosophila melanogaster*. Although greeted in the beginning by skepticism, this approach has led to some fundamental insights into mechanisms of learning and memory. The findings in *Drosophila* have converged with studies of *Aplysia* in identifying the cAMP-mediated signal transduction as a central pathway for learning and memory. The genetic studies of learning and memory have also been extended to other model systems, most notably, mice.

With modern techniques of genetics, many genes involved in learning and memory have been discovered in *Drosophila*, characterizations of which have given us considerable understanding of learning and memory mechanisms. The success of genetics in embryonic development is being played out in studies of learning and memory. In this chapter, we review how genetic approaches have been applied to dissect the processes of learning and memory in *Drosophila melanogaster* and use them to exemplify the role of genetics in learning and memory research.

II. GENETIC SCREENING OF LEARNING AND MEMORY MUTANTS

A. Behavior Measures of Learning and Memory

As important as a readily identifiable trait is for developmental studies, a simple and reliable behavioral assay is critical for identifying abnormal learning and/or
FIGURE 3-1 The T-maze assay for Pavlovian olfactory learning and memory in *Drosophila*.  
**A. Training:** About 100 flies are sequestered in a training tube whose inner surface is covered by an electrifiable grid. Odor currents can be drawn through the tube using a vacuum source attached to chamber a in the central elevator. The flies are first exposed to odor 1 while electric current is delivered to the grid and then to odor 2 with the current switched off. Such training can be repeated without an interval (massed training) or with an interval (spaced training).  
**B. Testing:** After training, the flies are transferred to chamber b in the central elevator to a choice point where currents of odor 1 and 2 are drawn in through two opposite testing tubes by vacuum attached to chamber b. If they have learned, the flies will avoid odor 1, which is associated with electric shock. Such performance can be quantified by the distribution of flies in the two testing tubes. Performance measured immediately after training is regarded as learning and at later time points as memory.

memory defects in large populations of flies. This challenge was met by Benzer and his colleagues Quinn and Harris at the California Institute of Technology, who developed an olfactory behavior test that paired electric shock with an odor cue, allowing assessment of associative learning in flies. Subsequently, Tully and Quinn (1985), then at Princeton University, developed a Pavlovian olfactory learning assay (Fig. 3-1) that allows direct comparisons of the behavioral properties of learning and memory among *Drosophila* and other invertebrate and vertebrate species. In this assay, flies are exposed to two odors. During exposure to one of them, the flies are given an electric shock. Such training results in a strong avoidance of the odor that is accompanied by electric shock and, consequently, robust memory retention. Flies can remember up to one day after a single training session, and four days after 10 successive training sessions without any interval. The memory can be further extended to a week if the flies are trained for 10 sessions with proper intervals (Tully et al., 1994).

**B. Chemical Mutagenesis**

The initial screening was carried out by feeding flies the chemical mutagen ethylmethane sulfonate (EMS). EMS is an alkylating agent that adds an alkyl group to the guanine bases of DNA. On replication, the modified guanine (G) base will pair with thymine (T) instead of the preferred cytosine (C) base, leading to point mutations from G:C to A:T. Other chemical mutagens,
including N-ethyl-N-nitrosourea (ENU), also directly modify DNA bases, resulting in point mutations. With such an approach, Benzer's group at Caltech identified the first single-gene mutant for associative learning, *dunce* (dnc) (Dudai et al., 1976). Continuing efforts by Quinn's group at Princeton isolated *rutabaga* (rut) (Livingstone et al., 1984), *amnesiac* (ann) (Quinn et al., 1979), *radish* (rsh) (Folkers et al., 1993), *cabbage* (cab) (Aceves-Pina et al., 1979), and *turnip* (tur) (Choi et al., 1991).

Chemical mutagenesis offers a high mutation rate and a broad target range. Chemical mutagens usually have low target specificity, and therefore the whole genome can be targeted for their mutational effects. The point mutations induced by chemical mutagens generate a diverse range of alleles, sometimes with conditional alleles (for example, thermosensitive alleles), which can provide more informative insights into the functions of a gene. One limiting factor for this approach is that the affected genes are hard to clone.

### C. Transposon Mutagenesis

The transposon mutagenesis is achieved by random transposition of transposons, mobile DNA elements (P-elements in *Drosophila*). Stripped of their autonomous ability to transpose, engineered P-elements can be stably integrated into the *Drosophila* genome and remobilized simply by crossing the P-element-carrying fly with one possessing a transgenic transposase activity (Rubin and Spradling, 1982; Cooley et al., 1988). The mobilized P-element can insert into random chromosomal loci. Genes neighboring the P-element insertion sites are often disrupted. Compared to chemical mutagenesis, affected genes in transposon mutagenesis can be easily identified with the help of the P-element tag (Cooley et al., 1988). The limitation is that in transposon mutagenesis, complete loss-of-function alleles are typically generated and the mutations are biased by the sequence specificity of the transposon. With this method, three additional learning mutants were isolated: *latheo* (lat) (Boynton and Tully, 1992), *linotte* (lio) (Dura et al., 1993), and *nalyot* (nal) (DeZazzo et al., 2000).

A variant of the transposon approach utilizes the enhancer-trap technique (O'Kane and Gehring, 1987), which in addition to producing mutagenesis, allows quick identification of the expression pattern of the affected gene. It incorporates in the P-element a reporter gene, *lacZ*, whose expression generally reflects the nearby gene and which can be revealed by β-galactosidase staining. Screening was performed using the enhancer-detection method to identify genes showing preferential expression the mushroom body (MB), a brain structure critical for olfactory learning and memory (Han et al., 1996). Among these genes, *leonardo* (leo) (Skoulakis and Davis, 1996), *volado* (vol) (Grotewiel
et al., 1998), and fasciclin II (fasII) (Cheng et al., 2001) are found to be involved in olfactory learning and memory. This approach also revealed that dnc, rut, and DCO (see later) are preferentially expressed in the MB. A cautionary note is that preferential expression does not necessarily imply the region of relevant gene function. For example, rut is also required for visual memory in another structure of the brain (Liu et al., 2006), although its expression is enriched in the MB.

Most recently, Tully's group at Cold Spring Harbor Laboratory used the enhancer-trap approach and screened for mutants with deficits in one-day memory after spaced training (Dubnau et al., 2003b). Sixty mutant strains were identified, and the P-element insertion sites were defined for 58 of those. It is revealed that in 28 mutant strains, the P-element landed in 25 known transcriptional units (three of these genes were hit twice). One of these transcriptional units is pumilio (pum), a transcript-specific translational repressor. Notably, pumilio was also uncovered in a DNA microarray screening (see Section D). A similar screening effort for identifying long-term-memory mutants uncovered crammer (cer), which might function as a transinhibitor of cathepsins (Comas et al., 2004).

D. DNA Microarray Screening

Instead of working on one gene at a time, DNA microarray offers the opportunity to monitor a whole genome simultaneously on a chip. Dubnau's group at the Cold Spring Harbor Laboratory used this technology and identified genes that are transcriptionally regulated during long-term-memory formation in normal flies (Dubnau et al., 2003b). Among those are pumilio, which is also identified in the aforementioned mutagenesis screening, and a few others, including staufen (stau), orb, and eIF-5C, which are known to be involved in local control of mRNA translation.

E. Anatomical Screening

Heisenberg's group at the University of Würzburg, Germany, screened for single-gene mutants with gross anatomical defects in various regions of the adult brain. Minibrain, a mutation that reduces the size of the adult brain, and several mutations affecting the mushroom bodies (MBs) or central complex (CC) — namely, mushroom body miniature, mushroom body deranged, central body defect, ellipsoid body open, central complex deranged, central complex broad, and no bridge — also show olfactory learning defects (Heisenberg et al., 1985; R.L. Davis, 1996; de Belle and Heisenberg, 1996).
F. Reverse Genetics to Identify Learning and Memory Mutants

While forward genetics starts from a phenotype and moves toward identification of the mutated gene, reverse genetics goes in the opposite direction. It starts with the knowledge of a gene sequence. Then the gene is disrupted or modified through targeted mutagenesis, followed by examination of the ensuing phenotypes to determine the gene's function. In *Drosophila*, several systematic gene-disruption projects using P-element insertion have generated thousands of stocks that each harbor a single P-element construct inserted at a known location in the genome. In addition, mutations in a known DNA sequence can be generated by homologous recombination (Rong and Golic, 2000; Rong et al., 2002) or by combining chemical mutagenesis and methods that detect single-nucleotide polymorphism (Stemple, 2004; Winkler et al., 2005). Aided with knowledge derived from molecular identification of the learning and memory mutants generated from mutagenesis screenings, gene discovery can be facilitated in reverse-genetics approaches by examining ready-made stocks with mutations in genes of interest for learning and memory defects. For example, biochemical analysis of *dunce* and *rutabaga* suggested that the cAMP signal transduction cascade is involved with olfactory learning. This discovery prompted a focused analysis of learning in extant mutants of *DCO* (the catalytic subunit of the cAMP-dependent protein kinase, PKA), *Su-var*(3) (a type I protein phosphatase PPl), and *Shaker* (R.L. Davis, 1996; Waddell and Quinn, 2001). Moreover, reverse-genetic disruptions of *Ga*, *PKA-RI* (a type I regulatory subunit of PKA) and *dCREB2* (cAMP-responsive element binding transcription factor) revealed roles for these genes in olfactory associative learning and memory (Waddell and Quinn, 2001; Dubnau et al., 2003a).

Other reverse-genetics strategies include direct manipulation of the gene of interest. The reverse-genetics approach from studies of human neurodegenerative disorders has led to identification of *neurofibromatosis 1* (*NF1*) and *nebula* (*nla*) as learning and memory genes (Guo et al., 2000; Chang et al., 2003). Neurofibromatosis type 1 (NF1) is a common human genetic disorder that exhibits tumors in the nervous system and learning and memory defects. It is caused by mutations in the *NF1* gene (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). Down's syndrome (DS) is the most common cause of mental retardation. Studies in DS patients have revealed a segment of chromosome 21, the DS region, that is closely related to phenotypic features of the DS. One of the genes in the DS region is the DS critical region 1 (*DSCR1*) gene. The cognitive defects in both disorders prompted interests in examining possible roles of *NF1* and *DSCR1* in learning and memory in *Drosophila*. It has turned out that both genes are involved in learning and memory. *Drosophila NF1* mutants show learning defects that could be rescued by conditioned expression of the *NF1* transgene (Guo et al., 2000). *Nebula* is a *Drosophila*
homolog of DSCR1. Mutations in nla produce learning and long-term memory defects that can be rescued by conditioned expression of an nla transgene (Chang et al., 2003).

G. Essential Controls

Critical to the characterization of single-gene mutations involved with learning is proper assessment of sensorimotor responses. This issue arises from the fact that learning per se is not observed directly but is inferred from a change in behavioral response after exposure to a stimulus. Hence, alterations in factors such as perception of the stimuli, fatigue, and motivational state also can affect behavioral responses. Single-gene mutants often have pleiotropic effects on many different behavioral responses, which also can make it difficult to determine if a given gene is involved with learning per se. A resolution to these problems lies first in determining which behavioral responses are pertinent to a given learning task and then in developing task-relevant assays for the sensorimotor responses required for normal performance. For Pavlovian olfactory conditioning, olfactory acuity and shock reactivity assays quantitate the flies’ abilities to sense and respond to the odors and shock stimuli used in the conditioning procedure. When a particular mutant fails these sensorimotor assays, one cannot conclude that poor performance in conditioning experiments results from a defect in learning per se. Conversely, when a learning mutant passes these sensorimotor tests, one gains confidence that the corresponding gene is involved in learning, regardless of any pleiotropic effects it may have on other behaviors. Of the genes mentioned earlier, seven (six affecting CC anatomy and tur) fail the task-relevant assays of sensorimotor responses required for Pavlovian olfactory learning. This highlights the importance of assaying performance in task-relevant sensorimotor responses.

III. GENETIC MANIPULATION OF CANDIDATE LEARNING AND MEMORY GENES

Unlike anatomical screens, behavioral screens for learning mutants can identify genes involved in both the development of neuronal structures and the biochemical pathways underlying behavioral plasticity of adults. Distinguishing these roles cannot be accomplished simply by analyzing gross neuroanatomical structure in the adult. Furthermore, to understand the underlying neural circuits of learning and memory, it is critical to know when and where in the brain the function of a candidate gene is required. Therefore, a candidate gene needs to be manipulated both in time, to rule out possible developmental effects and to determine its temporal requirement, and in space, to identify
the neural correlates of learning and memory. The temporal manipulation may be accomplished if fortuitously there exist conditional alleles of the gene of interest. This is usually through a temperature-sensitive mutant allele, whose function is disrupted at restrictive temperatures. Generally, the temporal and spatial controls over expression of a cloned gene can be achieved separately by the heat-inducible system and the GAL4-UAS system.

The temporal control in the heat-inducible system is conferred by a promoter from the *Drosophila heat-shock protein 70 (hsp70)* gene. The heat-inducible hsp70 promoter is cloned into a P-element vector upstream of a transgene to be expressed, and together they are incorporated into the fly genome following germ-line transformation. Expression of the transgene is controlled by the hsp70 promoter, which can be induced by heat. Hsp70-driven expression is global without any spatial specificity.

The GAL4-UAS system (Brand and Perrimon, 1993), which is based on the yeast GAL4 transcription factor and its upstream activating sequence (UAS), provides excellent spatial specificity for transgene expression (Fig. 3-2A). Analogous to an enhancer element in multicellular eukaryotes, UAS is essential
for the transcriptional activation of GAL4-regulated genes. GAL4 does not activate native *Drosophila* genes but can drive expression of a transgene placed downstream of UAS. GAL4, constructed in the same way as in an enhancer trap, respond to neighboring enhancers, and its expression often reflects that of the neighboring gene. Thus, the GAL4-driven transgene can be expressed in the same cell population or tissue as an endogenous *Drosophila* gene next to the GAL4 insertion site. Many fly lines with specific expression of GAL4 in various tissues and cell types have been generated. Additionally, lines carrying various transgenes fused with a UAS promoter have been established.

However, the GAL4-UAS system does not provide a temporal control. This can be achieved with a GAL4 under the control of the hsp70 promoter (Brand et al., 1994), but the spatial specificity is lost. To capitalize on the great collection of GAL4 and UAS lines and at the same time allow a temporal control, several systems have been developed with modifications of the GAL4-UAS system. They include the GeneSwitch, the tetracycline-inducible system, and TARGET (Osterwalder et al., 2001; Roman et al., 2001; Stebbins and Yin, 2001; S.E. McGuire et al., 2003). In all three systems, the spatial control is conferred by the GAL4-UAS system, but the eventual expression of the transgene is dependent on either feeding/withdrawing of specific substances, such as in GeneSwitch and the tetracycline-inducible system, or on elevation of temperature, such as in TARGET (Fig. 3-2B), and therefore a temporal handle is added onto the GAL4-UAS system. However, these systems usually take a day or days to attain the behavioral effect of transgene expression. So far, the heat-shock-induced transgene expression remains the best choice for the temporal control.

With these tools in hand, a gene of interest can be manipulated in many ways. Pertinent to the demonstration of a gene's involvement in learning and memory, one would naturally like to disrupt its function in time and space and examine the impact on learning and memory. This type of manipulation can be achieved by expression of a transgene dominant-negative to the endogenous gene or by RNA interference (RNAi). While the dominant-negative transgene product will compete in vivo with the endogenous protein and interrupt its function, transgenic expression of double-stranded RNA triggers an enzymatic degradation of the homologous endogenous mRNA, thus producing gene-specific posttranscriptional silencing (Lam and Thummel, 2000; Zamore et al., 2000). Another type of manipulation involves reintroducing a wild-type transgene into the mutant fly strain. Rescue of the mutant learning and memory defect by the expression of the transgene can provide ultimate evidence that the right gene has been identified from the mutant. Further insight into the involvement of the gene in learning and memory can be gained from the temporal and spatial requirement of the transgene expression for the rescue.
IV. GENETIC DISSECTION OF LEARNING AND MEMORY

Genetic tools provide powerful means to study learning and memory. Genetic screening has enabled unbiased discovery of gene mutations affecting learning and memory. The identification of these genes and subsequent reverse-genetics manipulation have allowed dissection of learning and memory at the molecular, synaptic, and systems levels.

A. Dissection of Biochemical Pathways

The behavioral and molecular studies of the mutants dnc, rut, and amn have suggested that signal transduction mediated by cAMP plays a key role for learning and memory in the fly. dnc, rut, and amn loci encode, respectively, a phosphodiesterase that degrades cAMP, an adenylyl cyclase (AC) that converts ATP to cAMP, and a peptide transmitter that stimulates AC. Reverse genetics has shown that other players in the cAMP pathway all affect learning and memory.

For dnc, rut, DCO, and dCREB2, inducible transgenes and conditional alleles have been used to demonstrate an acute role for these genes in the biochemistry of adult associative learning (Drain et al., 1991; Yin et al., 1994; Dauwalder and Davis, 1995; Yin et al., 1995; Li et al., 1996; McGuire et al., 2003). Induced expression of a transgene encoding a dnc$^{+}$ cDNA yields partial rescue of the learning defect in dnc$^{-}$ mutants, revealing a role for dnc during adult associative learning. In the absence of complete rescue, however, it remains possible that the residual learning defect results from anatomical defects that arise during development. An acute role for rut in memory is demonstrated with the TARGET system (S.E. McGuire et al., 2003). Transient expression of the rut-encoded AC in the adult is necessary and sufficient to rescue the rut mutant memory defect, while expression during development is not.

DCO, the catalytic subunit of PKA, also has been shown to function in learning and memory in the adult. Pavlovian olfactory learning is reduced in heteroallelic combinations of DCO$^{581}$ and DCO$^{R10}$ (Skoulakis et al., 1993); memory retention three hours after training, however, is normal in these mutants. Induced expression of transgenes encoding either the catalytic subunit of PKA, a peptide inhibitor of PKA, or a truncated mammalian type II regulatory subunit (RII) of PKA (in which the cAMP binding site was removed) causes a decrease in Pavlovian olfactory learning (Drain et al., 1991). In contrast, transgenes expressing full-length RII subunit have no effect. Task-relevant sensorimotor responses are normal before or after heat shock in each of these transgenic lines and in the heteroallelic mutants. These findings demonstrate an acute role for PKA in adult olfactory learning.
Evidence for an acute requirement for PKA activity during memory formation after Pavlovian olfactory learning comes from studies of DC0^vy, a conditional lethal allele. At the permissive temperature, DC0^vy/Df hemizygous mutants show a reduction in learning, but they have normal memory decay. At the restrictive temperature, however, DC0^vy/Df hemizygous mutants show the same learning defect and a disruption of middle-term memory (Li et al., 1996). This study suggests that different thresholds of PKA activity may be required for development, learning, and memory formation.

More insights into cAMP signaling have come from analyses of a neuropeptide known as pituitary AC activating peptide (PACAP) and the Drosophila homolog of the human neurofibromatosis type 1 (NFl) gene. In vertebrates, PACAP activates AC through a G protein-coupled receptor. In Drosophila, application of mammalian PACAP38 to the neuromuscular junction (NMJ) results in a slow inward current lasting tens of seconds, followed by an enhancement of outward K^+ current. This PACAP-induced response is impaired in rut mutants (Zhong, 1995; Zhong and Pena, 1995), suggesting activation of AC by PACAP. Concomitant activation of the Ras and cAMP signaling pathways is required to mediate PACAP function.

In mammals, NFl is believed to function as a GTPase-activating protein for Ras (Ras-GAP) and therefore as a negative regulator of Ras (Ballester et al., 1990; Buchberg et al., 1990; Martin et al., 1990; Xu et al., 1990a, 1990b). In flies, mutations in NFl eliminate the PACAP response. Application of either cAMP analogs or forskolin (which stimulates G protein-coupled AC) restores the PACAP response in NFl mutant flies, suggesting that NFl regulates rutabaga-encoded AC (Guo et al., 1997). G protein-stimulated AC activity is reduced in NFl mutants and can be restored by both Drosophila and human NFl transgenes (Guo et al., 2000; Tong et al., 2002). Moreover, overexpression of PKA is sufficient to rescue developmental defects of NFl mutant flies (The et al., 1997).

Three distinct AC signaling pathways in Drosophila have now been identified, including a novel growth factor-activated NFl/Ras-dependent AC pathway, as well as two separate neurotransmitter-stimulated AC pathways (Fig. 3-3) (Hannan et al., 2006). The growth factor–stimulated AC pathway can be disrupted by mutations in the epidermal growth factor receptor (EGFR), NFl, and Ras but not Go. The second AC pathway is stimulated by serotonin and histamine requiring NFl and Go. The third is a classical Go-dependent AC pathway, which is stimulated by Phe-Met-Arg-Phe-amide (FMRFamide) and dopamine. The NFl-dependent Rut-AC is required for learning (Guo et al., 2000).

Further study of the cAMP signaling cascade components, along with information about other learning/memory genes, suggests the involvement of additional biochemical pathways. Amorphic alleles of rut, for instance, reduce but do not eliminate olfactory learning (Livingstone et al., 1984). This suggests
the involvement of additional cyclases or of novel signaling pathways. The rsh mutant is deficient in anesthesia-resistant memory, a form of long-term memory in Drosophila, and it has been recently found that rsh encodes a protein with 23 predicted PKA phosphorylation sites (Folkers et al., 2006). In addition to genes involved in the cAMP signaling cascade, mutations in other pathways have been identified. In the learning mutant tur, protein kinase C (PKC) activity is significantly reduced compared with activity in wild-type flies (Choi et al., 1991). Moreover, an atypical PKC is required for a specific phase of memory (Drier et al., 2002). Ca\textsuperscript{2+}-CaM-dependent protein kinase II (CaMKII) is involved in courtship learning (Griffith et al., 1993). The learning mutant gene leo encodes a Drosophila homolog of the vertebrate 14-3-3\textsuperscript{c} isoform, a protein that is known to interact with diverse signaling proteins, including RAF-1, in the mitogen-activated protein kinase (MAPK) pathway and PKC (Skoulakis and Davis, 1996). A role for cell adhesion is demonstrated by the discovery of two mutants of cell adhesion molecules, fasciclin II and integrin,
that are defective in learning and memory (Grotewiel et al., 1998; Cheng et al., 2001). The characterization of additional learning and memory genes, such as *nal* and *lat*, which respectively encode the Adfl transcription factor (DeZazzo et al., 2000) and a subunit of the origin recognition complex that is involved in DNA replication (Pinto et al., 1999), and findings of requirement of *Notch* in long-term memory (Ge et al., 2004; Presente et al., 2004) suggest that still more biochemical pathways may be involved. These multiple signaling cascades may act in series or in parallel; they may act in the same neurons or in different brain structures; and they may contribute to different aspects of the learning and memory process.

**B. Dissection of Neuroanatomical Pathways**

The insect brain contains several prominent neuropilar structures (Fig. 3-4). MBs are large bilaterally symmetric structures that are positioned in the dorsal/posterior region of the central brain. In adult *Drosophila*, MBs consist of ~2,500 neurons (Kenyon cells) in each brain hemisphere. The MB neurons can be divided into three types, the γ, α/β', and α/β neurons, depending on their
birth orders, and they give rise to separate axonal bundles (Crittenden et al., 1998; Lee and Luo, 1999). Axons of the γ neurons form the horizontal γ lobe, while those of α'/β' and α/β neurons bifurcate and extend into vertical α' and α and horizontal β' and β lobes, respectively. The primary olfactory inputs are conveyed from the antennal lobe (AL) along the inner antennal-cererebral tract (iACT) to the MB and to the lateral horn (LH) of the protocerebrum (Stocker et al., 1990). The LH also receives direct input from the AL along the outer and medial ACT (oACT, mACT). In addition to the cholinergic input that conveys olfactory information to the MB, there are dopaminergic and octopaminergic inputs to MB neurons. The dopaminergic neurons, which innervate both the calyx (dendritic field) and lobes (axon terminals) of the MB, are required for association of odor with electric shock, while octopaminergic innervation of MB underlies association of odors with sugar in an appetitive conditioning procedure (Schwaerzel et al., 2003). The dopaminergic and octopaminergic neurons may carry the US information in the associative learning. This is supported by the finding that dopaminergic neurons respond to electric shock experienced during learning (Riemensperger et al., 2005).

Convergent data demonstrate that MBs play an important role in olfactory associative learning in Drosophila (Heisenberg, 2003). Analyses of learning in mutants with defects in brain structure indicated that the MBs are required for olfactory (but not visual) learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1996). Chemical ablation of the MB or targeted disruption of the cAMP signaling cascade with a constitutively active Gaα (Gaα*) in the MB abolishes olfactory learning with no effects on the “task-relevant” sensorimotor responses (de Belle and Heisenberg, 1994; Connolly et al., 1996). Associative learning is normal when activated Gaα* is expressed in CC (Connolly et al., 1996). Expression of the rut-encoded AC in the MB rescues the rut memory defect (Zars et al., 2000; S.E. McGuire et al., 2003), further demonstrating a critical role for cAMP signaling within the MB, at least in early olfactory memory. This is consistent with the finding that multiple components of the cAMP signaling cascade (AC, PDE, PKA catalytic subunit, and RI regulatory subunit) are expressed at high levels in MBs (Han et al., 1996). Temporary disruption of MB neuron output with expression of a temperature-sensitive dominant-negative dynamin transgene, shibire, blocks memory retrieval (Dubnau et al., 2001; S.E. McGuire et al., 2001). Taken together, these findings support the idea that MB Kenyon cells are part of an anatomical circuit underlying olfactory learning and memory. In particular, the anatomical “circuit diagram” as well as the genetic perturbations of cAMP signaling in MB support a model in which MB Kenyon cells are at least one anatomical site where the US (electric shock) and CS (odors) are associated.

Results from studies of the amnesiac mutant, which is defective in memory but normal in learning, indicates that neurons outside the MB may also be
part of the neural circuits underlying learning and memory (Waddell et al., 2000). Amn encodes a PACAP-like peptide, and its expression is restricted to the dorsal paired medial (DPM) neurons, a pair of neurons that innervate the axon terminals of MB neurons. Developmental expression of an amn transgene in DPM neurons rescues (DeZazzo et al., 1999; Waddell et al., 2000), whereas transiently blocking DPM neuron output with the temperature-sensitive shibiri mimics the amn mutant memory defect without affecting learning, establishing a link between the DPM neurons and amn-dependent memory (Waddell et al., 2000).

A recent forward enhancer-trap mutagenesis screen for genes affecting long-term memory has also revealed the involvement of structures other than the MBs in long-term memory (Dubnau et al., 2003b). Some 60 new transposon-insertion mutants with one-day memory defect after spaced training are identified in this screening. Not surprisingly, about 60% of the mutants yield reporter expression in the MB. But more interestingly, a significant number of the mutants show expression exclusively outside the MB. One such mutant, murashka, shows restricted expression in a few neurons that innervate the MB calyx. Genetic manipulation of these newly found candidate genes in both time and space will certainly help us to construct the complete neural circuitry underlying olfactory learning and memory.

The CC is another prominent brain structure situated in the center of the supraesophageal ganglia. It comprises four substructures: the ellipsoid body, the fan-shaped body, the nodulii, and the protocerebral bridge (Fig. 3-4). The CC forms intricate connections to a variety of brain centers, is believed to be a control center for many different behavioral responses (motor output), and receives prominent, but not exclusive, visual input (Strauss, 2002). CC participates in visual memory as mutants with CC structural abnormalities are impaired in visual pattern memory (Liu et al., 2006). Intact rut gene is also required for visual-pattern memory. Expression of the wild-type rut cDNA (UAS–rut+) in CC rescues the visual memory deficit in the mutant rut flies. Strikingly, memories of different visual features — the angular orientation of a bar and its vertical position on the fly's retinal field — require rut-AC in different subsets of CC neurons.

C. Dissection of Synaptic Plasticity

Because of the remarkable biochemical and pharmacological similarities between mechanisms of cellular and behavioral plasticity, modifications of synaptic strength and structure are widely believed to underlie learning and memory (Kandel and Abel, 1995). Physiological and neuroanatomical studies of synaptic plasticity in learning and memory mutants have strengthened this notion considerably.
Several mutants show defective synaptic structure and function at the larval NMJ. Mutations in either eag or Sh (which encode K⁺ channel subunits), for instance, produce an altered K⁺ conductance, and eag-Sh double mutants show higher baseline activity and evoked hyperexcitability and decreased expression of the cell adhesion molecule, fasciclin II, resulting in increased synaptic arborization (Budnik et al., 1990; Zhong and Wu, 1991; Zhong et al., 1992; Schuster et al., 1996). dnc mutants also show increased neuronal activity and synaptic arborization, and these defects are enhanced in dnc-Sh double mutants (Zhong et al., 1992). Moreover, the synaptic defects in dnc-Sh and dnc-eag double mutants are suppressed by rut in triple mutant combinations. At the larval NMJ, then, cAMP signaling modulates synaptic structure in an activity-dependent manner.

Both mutant Vol (encodes a-integrin protein) and lat are impaired in multiple forms of synaptic plasticity at the NMJ (Rohrbough et al., 1999, 2000). In addition to functional abnormalities, Vol mutant synaptic arbors are structurally enlarged, suggesting Volado negatively regulates developmental synaptic sprouting and growth. In the leo mutant NMJ, basal synaptic transmission is reduced by 30% and transmission amplitude, fidelity, and fatigue-resistance properties are reduced at elevated stimulation frequencies and in low external [Ca²⁺] (Broadie et al., 1997). Moreover, transmission augmentation and posttetanic potentiation (PTP) are disrupted in the mutant. These results suggest that Leonardo plays a role in the regulation of synaptic vesicle dynamics.

Overexpression of CREB repressor suppresses the increase in neuronal activity but has no effect on the increased arborization, produced by the dnc mutation (G.W. Davis et al., 1996). Moreover, overexpression of CREB activator alone does not appear to alter structure or function at the larval NMJ but does produce an increase in synaptic function in Fas II mutants, which normally show increased arborization without an increase in activity. On the other hand, reduced expression of Adf1 in the mutant nal and heat-shock-induced overexpression of the wild-type Adfl gene have opposite effects on synaptic structure, but neither affects synaptic function (DeZazzo et al., 2000). These data suggest that activity-dependent modulation can be dissected into a CREB-mediated functional pathway and an Adf1-mediated structural pathway. AP-1, a heterodimeric transcription factor composed of Fos and Jun, regulates both synaptic strength and synapse number (Sanyal et al., 2002). Overexpression of dnc phosphodiesterase fails to block the influence of AP-1 on synapse number. Conditioned overexpression of AP-1 results in robust increase in CREB mRNA levels. These observations indicate that AP-1 acts upstream of CREB.

NMDA receptor (NMDAR) plays an important role in experience-dependent synaptic plasticity, including long-term potentiation and long-term depression (Bear, 1996). Because of its unique property of voltage-dependent activation by ligand, NMDAR has been suggested as a "Hebbian coincidence
detector" underlying associative learning. Mutations of the Drosophila NMDAR gene cause learning defects that can be restored by wild-type transgenes (Xia et al., 2005). Acute disruption of NMDAR with an NMDAR antisense RNA transgene impairs learning and long-term memory formation, providing direct evidence for acute involvement of NMDAR in associative learning and memory.

D. Dissection of Memory Formation

Memory formation also represents a signaling pathway of sorts — from learning to long-term memory. Behavior-genetic analyses of this process have suggested five distinct temporal phases: long-term memory (LTM), anesthesia-resistant memory (ARM), middle-term memory (MTM), short-term memory (STM), and acquisition (or learning (LRN)). Single-gene-mutant analyses indicate that memory processing is sequential from LRN to MTM but that consolidation of ARM and LTM then occurs in parallel (Fig. 3-5).

1. LTM

LTM is protein synthesis dependent, appears within 24 hr after spaced training (10 training sessions with a 15-min rest interval between each), and lasts for more than one week. In contrast, LTM is not produced after massed training (10 training sessions with no rest interval between each) (Tully et al., 1994). LTM is blocked by protein synthesis inhibitors and depends critically on gene expression mediated by Adfl, CREB transcription factors, and Notch signaling (Yin et al., 1994, 1995; DeZazzo et al., 2000; Ge et al., 2004; Presente et al., 2004).

2. ARM

Memory is initially labile but eventually can be consolidated into more stable forms. Memory soon after Pavlovian olfactory learning, for example, is disrupted by cold shock. During the first two hours after training, however, a cold-shock-insensitive form of memory (or ARM) appears (Tully et al., 1994). ARM is the longest-lasting memory produced by massed training, appears to be protein synthesis independent, and decays away within four days. Although multiple training cycles do produce increasingly higher levels of ARM, 10 massed and 10 spaced training sessions both yield similar maximal levels of ARM (Tully et al., 1994). ARM is unaffected by inhibition of protein synthesis or by disruptions of Adfl, CREB, or Notch transcription factors that block LTM (Yin et al., 1994; DeZazzo et al., 2000; Ge et al., 2004; Presente et al., 2004), but it is disrupted in rsh mutants (Tully et al., 1994). In contrast,
FIGURE 3-5  Distinct memory phases. A. Four functionally distinct memory phases have been detected underlying the observed memory retention scores. Short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and long-term memory (LTM) appear sequentially, and each has a progressively slower rate of decay. B. A genetic model of information flow showing where in the pathway single-gene mutants have their primary effects. The pathway is sequential from learning (LRN) to MTM, but then it branches into two independent paths — one leading to ARM and the other leading to LTM. (Reproduced with modifications from Dubnau and Tully, 1998.)

LTM in rsh mutants is normal. Thus, ARM and LTM are genetically and functionally independent forms of long-lasting memory that exist in parallel for several days after spaced training. A study on mutant flies devoid of MB vertical lobes (ala) suggests that ARM and LTM are exclusive to each other (Isabel et al., 2004). It has to be cautioned that ala is a developmental mutant and that the abnormal memory formation in ala may be a result of aberrant wiring of the MB (Margulies et al., 2005).

3. MTM

Two hours after training, when ARM is maximal, approximately 50% of observed memory is still cold-shock sensitive. This early memory is also resistant to protein synthesis inhibitors and can be further decomposed into STM and MTM by the amn mutation. Initial learning in amn is near normal, as is
7-hr memory retention. Memory retention at intermediate time points, however, is reduced. This observation first suggested the existence of MTM.

Evidence for MTM in wild-type flies emerged from reversal retention experiments (Tully et al., 1990, 1996). During the first training session, odor A (the CS+) is paired with electroshock, and odor B (the CS-) is not. In a second training session, this arrangement is reversed: Odor B becomes the CS+, and odor A becomes the CS-. Different groups of flies are then subjected to reversal learning at various time points after the first training session. In each case, however, conditioned responses are quantified immediately after the second training session. A temporal window is revealed, within which the first odor-shock association is sensitive to disruption by the reversal learning. Strikingly, the disrupted memory appears to correspond quantitatively and temporally to that missing in amn mutants. Moreover, the asymptotic level of reversal learning-resistant memory is quantitatively similar to that of ARM. These observations suggest that reversal training disrupts a memory component in wild-type flies that is already genetically impaired in amn mutants, thereby eliminating any difference between wild-type and amn reversal retention curves. In fact, the reversal retention curves of wild-type and amn flies are indistinguishable. These data suggest that MTM may be a genetically distinct component of memory.

Study on a temperature-sensitive DCO mutant (DC0') also supports the existence of a genetically distinct MTM phase (Li et al., 1996). DCO' has a learning defect, but shifting from permissive to restrictive temperature further disrupts memory retention. The temperature-shift-specific effect is indistinguishable from the amnesiac memory retention curve. Thus, disruption in DCO function in adult flies appears to disrupt MTM.

4. STM

Far less is known about STM. Memory decay within 30 min of training is faster in dnc and rut than in amn mutants or wild-type flies, suggesting that the former mutants disrupt a memory component that temporally precedes MTM (i.e., STM). Memory decay after the first 30 min slows considerably in dnc and rut, but retention levels in these mutants are clearly lower than those in amn. This observation raises the possibility that MTM is downstream of, and dependent on, STM.

5. LRN

In contrast to dnc and rut, lat, lio, PKA-R1, and fasII all show reduced Pavlovian olfactory learning, but the rate of memory decay thereafter appears normal (Dubnau and Tully, 1998; Cheng et al., 2001). Hence, these genes may be involved exclusively in the initial acquisition of the odor and shock association.
Learning defects traditionally have been suspected to result more often from mutations that disrupt development than from adult biochemistries. This is clearly not the case for fasII mutants, however, since induced expression of fasII transgenes in adults fully rescues the fasII learning defect. Mutants with normal (or near-normal) learning but defective memory decay, in contrast, have been thought to result more likely in biochemical defects. This generalization also is invalid. If memory retention requires distinct anatomical structures, for instance, then abnormal development might yield memory-specific behavioral defects. Thus, eliminating maldevelopment as a possible explanation for learning and memory defects in adults resides solely in the use of conditional mutations or inducible transgenes.

V. SUMMARY

The power of genetic dissection is limited only by the breadth of gene discovery. The ongoing discovery of genes involved with learning and memory stands to revolutionize our understanding of behavioral plasticity, in two ways. First, systematic analyses of the genetic, biochemical, and cell biological requirements of each gene will yield a vertical integration of information across biological levels of organization. Second, the evolutionary conservation of core mechanisms of cell signaling has enabled isolation of vertebrate homologs and transfer of some of the logic underlying invertebrate learning and memory to vertebrate systems, providing a horizontal integration of information across species and model systems. To that extent, the mouse, with its own versatile genetic toolboxes, is beginning to play a more and more important role in learning and memory research.

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