13

Memories of Worms and Flies: From Gene to Behavior

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THE POWER OF THE GENETIC APPROACH to biological questions derives from the ability to forge links between reductionist and holistic levels of organization, but the links are largely inferred until the underlying mechanisms have been delineated in detail. This strategy was first used to dissect relatively simple biological systems such as those controlling growth and metabolism of cells. Then, the vastly more complex systems underlying embryonic development came into the sights of geneticists, largely relying on a few model organisms such as "the worm," *Caenorhabditis elegans,* and "the fly," *Drosophila melanogaster*. The result has been an astounding revolution in our understanding of morphogenesis across phyla. But the success of this tactic rested on the prior work of meticulous classical embryologists who had so carefully observed and described the phenotype: the development of embryos. The success of developmental genetics thus relied on a prior understanding of much of the phenomenology of embryonic development.

Today, the same strategy is being used to tackle the astounding complexity of memory. Again relying on model organisms such as "the worm" and "the fly," geneticists are beginning to forge links between reductionist mechanism and holistic behavior. Once again, however, the success of this approach will hinge on our ability to follow the prior generation of

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behaviorists who so carefully characterized the phenotype, in this case, the psychology of memory.

In this chapter, we have attempted to provide a flavor for how this has proceeded so far and to give our vision for where this field will need to go. We have by no means tried to fully review the fields of behavior-genetic study of memory in worms and fruit flies. Instead, we have focused on key studies that in our view exemplify the reasons we believe the genetic approach ultimately will prove valuable in discovering how brains accomplish the task of remembering past experiences.

THE PSYCHOLOGY OF MEMORY: THE PHENOTYPE

Studies on the cellular basis of memory first gained popularity in the early 1970s with work on mollusks exemplified by the work of Eric Kandel and his colleagues (for reviews, see Hawkins et al. 1993, 2006; Glanzman 2006) in their studies of cellular mechanisms of learning and memory in *Aplysia*. *Aplysia* are excellent models for physiological analyses because they have relatively small nervous systems (~20,000 neurons) and large neurons that are easy to record from. Their research led to a number of insights into cellular mechanisms of simple forms of learning and memory. Although *Aplysia* are excellent subjects for physiological studies, they are not good candidates for genetic analyses. With the development of modern molecular genetics, finding model systems in which to study the genetic bases of learning and memory was an important step forward. The first such system to be developed was *Drosophila melanogaster*. When the first genetic mutations affecting learning and memory in *Drosophila* were shown to be in molecules already implicated in learning and memory in *Aplysia*, the convergence of the biochemical pathways for learning and memory in these two species was seen as validation for the model system approach. More recently, *Caenorhabditis elegans* has also been developed as a genetic model for learning and memory studies. Worms and flies each exhibit a wide array of stereotyped behaviors, but they also are capable of a remarkable degree of behavioral plasticity and perhaps even occasional displays of cognitive brilliance. Because these animals have long been developed as genetic model systems, they provide a unique opportunity for molecular genetic dissection of memory. A number of robust experimental assays of several different forms of memory already have been established in each of these animals.

The simplest forms of learning that organisms exhibit are nonassociative tasks in which the attention that an organism directs to an indi-

vidual stimulus is changed. Nonassociative learning includes habituation, in which an animal decreases its response after repeated or prolonged stimulation, and sensitization, in which an animal increases its response following a strong noxious stimulus. Both worms and flies are also capable of associative learning in which the animals learn a temporal, predictive relationship between two stimuli. Like nonassociative tasks, associative learning has been thought of in terms of two types of experimental paradigms: classical (Pavlovian) and operant conditioning (for a detailed description, see Mackintosh 1983). In Pavlovian learning, the animal is exposed to two stimuli: an unconditioned stimulus (US; the food in the famous case of Pavlov's dogs), which normally elicits an unconditioned response (UR) or reflex even in naïve animals (salivation in Pavlov's dogs), and a conditioned stimulus (CS; the bell in the case of Pavlov's dogs), which elicits a conditioned response (CR; salivation in the dogs) only after conditioning. Thus, the CS is the stimulus that the animals learn about. In Pavlovian conditioning, the experimenter temporally pairs the delivery of the two stimuli: the bell and the food reward. This typically causes an association between the stimuli resulting in the CR (salivation) during subsequent exposures to the CS (the bell). In Pavlovian learning paradigms, the nature, timing, duration, strength, and number of stimuli are entirely under control of the experimenter.

Regardless of the species or learning task, memory processing, storage, and retrieval are each remarkably dynamic. One of the hallmarks of memory is a progressive consolidation from initially labile forms of storage into forms that are highly resistant both to experimental manipulation and to the passage of time. Much research effort has focused on this transition from so-called short-term memory (STM) to long-term memory (LTM).

Most of the research on memory in *C. elegans* has been on STM; however, there are studies of LTM as well (for review, see Giles et al. 2006). Research in flies has focused on a dissection of genetic pathways of early memory as well as on consolidation of two different forms of long-lasting memory. Although worms and flies each are capable of both nonassociative and associative learning, we have focused this review on a nonassociative task in worms and on a Pavlovian task in flies. This is partly due to space limitations, but also owes to the fact that these tasks have been most exploited experimentally in worms and flies, respectively. These two tasks also exemplify the reasons that the study of learning requires carefully controlled experimental paradigms. From an experimenter's point of view, learning and memory are not directly measurable; instead,

they are inferred from a change in the performance of the animal caused by prior experiences. For this reason, controls are critical in order to rule out other factors that could affect performance such as deficits in sensory and motor systems or in motivation or arousal that can result from genetic manipulation, physical handling, or pharmacological treatments. The simplicity of the habituation assay in worms and the Pavlovian assay in flies permits direct measurement of these task-relevant responses.

In addition to the differences in behavioral assays, these two organisms also differ dramatically in the complexity of their nervous systems and in the experimental strengths that they offer. This almost certainly reflects differences in neural mechanisms utilized and has affected the types of questions that can best be answered with each model system.

THE ANIMALS

C. elegans

In the late 1960s, Sydney Brenner went looking for an ideal animal to study the genetic control of development and of nervous systems and behavior and chose *C. elegans* (Brenner 1974). *C. elegans* are small (1-mm) nematode worms whose normal habitat is soil, but they can easily be maintained on agar plates in the laboratory on a diet of *Escherichia coli*. An adult hermaphrodite can produce a large number of eggs (>300) by self-fertilization beginning at about 3.5 days of age.

C. elegans was the first multicellular organism to have its genome fully mapped and sequenced. The worm nervous system consists of 302 neurons; every neuron has been identified, its cell lineage determined, and its synaptic connectivity patterns mapped at the electron microscopic level (~5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions; White et al. 1986). This provides researchers with a complete set of "identified neurons" to study in their quest to understand behavior. An identified neuron is a recognizable cell that occurs in the same location and has the same function in every member of a species. It allows researchers to test the function and properties of the same neuron in a variety of animals under a variety of conditions. As shown throughout this volume, the use of invertebrate-identified neurons has led to many insights into physiological processes within neurons and has allowed researchers to establish causal relationships between changes in the nervous system and changes in behavior. Because the worm is transparent, noninvasive neural circuit analyses can be done using laser ablation of identified neurons (Chalfie et al. 1985). This has allowed

researchers to investigate the role of single neurons in specific behaviors and then use the circuit diagram to explore the roles of connected neurons in that same behavior. The worms' transparency has also led to development of genetic expression reporter constructs (i.e., green fluorescent protein [GFP]) (Chalfie et al. 1994) that permit visualization of gene expression in living animals, thus forging links from gene product to identified neuron and then to behavior.

As in all organisms, *C. elegans* neurons differentiate, send out processes that migrate to their targets, form synapses with the correct targets, and synthesize, package, and release neurotransmitters. Appropriate receptors on postsynaptic neurons directly or indirectly activate ion channels to transmit the signal to their terminals. Much of the cellular machinery used by *C. elegans* to carry out these processes is homologous to that known in other organisms. *C. elegans* uses acetylcholine, GABA, glutamate, serotonin, dopamine, and neuropeptides as neurotransmitters for synaptic communication (Jorgenson and Kaplan 2006).

At the behavioral level, *C. elegans* also share many similarities with animals from other phyla. They can respond to a variety of environmental stimuli including taste, smell, temperature, and touch; mutations altering each of these sensory systems have been studied (for review, see Rankin 2002). *C. elegans* shows a variety of forms of learning and memory that have been studied at behavioral, neural, and genetic levels (for review, see Giles et al. 2006). Two different approaches have been used to investigate genes involved in learning and memory. One is a classic forward genetics approach of screening large numbers of mutants in order to discover new genes that have a role in the phenotype. The other is the candidate gene approach in which a gene that has been identified previously is tested for its role in a particular phenotype. Both of these approaches have yielded insights into cellular mechanisms of learning and memory.

D. melanogaster

In the beginning, there was Morgan's "fly room" at Columbia University. There, T.H. Morgan and his students H.J. Muller, A.H. Sturtevant, and C.B. Bridges not only laid the foundation of genetics, but also sealed the fate of *D. melanogaster* as one of the chosen model organisms for its study. The 100 years of genetic research that followed has generated a staggering wealth of tools that aid in genetic dissection of virtually any phenotype. Although *Drosophila* is now most famous for the saturation screens for mutations affecting development of the embryo, "the fly" also

has become a playground for dissection of behavior beginning with Seymour Benzer's seminal work in the late 1960s. Benzer believed that the biological underpinnings of complex behaviors, like those of development, could be uncovered by isolation of single-gene mutations. This revolutionary idea has become the inspiration for using the genetic, molecular genetic, and now genomic tools available in *Drosophila* to investigate a diverse array of innate and learned behaviors. *Drosophila* genetic approaches have been used to investigate mechanisms of sensory perception, geotaxis, phototaxis, and pain (Carlson 1996; Vosshall 2000; Caldwell and Eberl 2002; Goodman 2003; Amrein and Thorne 2005); control of complex multimodally driven behaviors such as flight, walking, and foraging (Sokolowski 2001; Strauss 2002; Frye and Dickinson 2004); and gender dimorphic behaviors such as aggression (Chen et al. 2002) and courtship (Greenspan and Ferveur 2000). The genetic basis of circadian rhythms was discovered and largely worked out in flies (Hall 2003). Flies also have been used to study responses to and lasting behavioral adaptations to addictive drugs (Wolf and Heberlein 2003), genetic control of neurodegeneration (Bilen and Bonini 2005), visual attention (Swinderen 2005), sleep (Shaw and Franken 2003), and of course memory—the topic of this chapter. Behavioral assays of memory in flies include several for habituation and sensitization, appetitive and aversive classical conditioning, operant visual learning, operant spatial learning, courtship suppression learning, and age-dependent memory loss (Dubnau and Tully 1998; Gerber et al. 2004a; Mehren et al. 2004; Margulies et al. 2005; Saitoe et al. 2005).

Although the nervous system of *Drosophila* is often touted in grant proposals as a "simple model" (J. Dubnau, unpubl.)—which is true relative to vertebrate species—the fly brain is objectively speaking and relative to that of *C. elegans*, an astoundingly complex circuit. In contrast to the worm, which contains 302 neurons whose lineage and connectivity are largely invariant and fully mapped, this fruit fly possesses approximately 100,000 central nervous system neurons whose connectivity is largely unknown. Moreover, the beautiful invariance of lineage and wiring observed in the worm brain does not apply to the fly or to massively more complex brains of vertebrates. This has proven to be a double-edged sword. On the one hand, the thought of fully mapping the circuitry of the fly brain is a daunting one, but, on the other hand is the appeal of a genetic system to study the complexity of behavior and cognitive processing of which flies are capable.

C. elegans is the ideal "stripped down" nervous system amenable to investigation of fundamental questions about brain function. The

strengths of the worm for forward mutagenesis are unparalleled in the animal kingdom. But flies offer a somewhat different attraction: a more complex nervous system and a more diverse behavioral repertoire that still is amenable to genetic manipulation. In the following sections of this chapter, we review some of the literature that illustrates the types of contributions that genetic approaches in these two species have made to our understanding of memory.

HABITUATION OF THE TAP RESPONSE IN WORMS

The most basic nonassociative form of learning is habituation, decreased responding to a repeated (or long-lasting) stimulus (Groves and Thompson 1970). Despite the apparent simplicity of this for learning, remarkably little is known about the underlying cellular mechanisms. Habituation is a phenomenon of attention: It can be thought of as a filter that allows an animal to ignore irrelevant stimuli so that it can attend to stimuli that signal good (i.e., food) or bad (i.e., dangerous) factors. Habituation can be differentiated from sensory adaptation or motor fatigue in one of two ways: First, habituation can be rapidly reversed by the application of a novel or noxious stimulus in a process termed dishabituation (Groves and Thompson 1970). Second, the rate of spontaneous recovery from habituation is dependent on the rate of the stimuli during habituation; recovery following habituation at high frequencies is more rapid than that following habituation at lower frequencies (Rankin and Broster 1992).

The best-studied habituation in *C. elegans* is habituation of the tap-withdrawal response, a mechanosensory response of swimming backward in reaction to a tap delivered to the side of the petri plate holding the worm (Rankin et al. 1990; for review, see Giles et al. 2006). In response to the tap, the worm swims backward for a brief distance; this is called a reversal. Using laser ablation of individual identified neurons and then testing the behavior of the ablated worms, Wicks and Rankin (1995) showed that this response is mediated by a simple neural circuit made up primarily of five mechanosensory neurons (ALML, ALMR, and AVM in the head and PLML and LPMR in the tail) and four pairs of interneurons (AVA, AVB, AVD, and PVC) (see Fig. 1).

C. elegans shows both STM and LTM for habituation to tap. Short-term habituation differs depending on the interstimulus interval used. Short interstimulus intervals (ISIs; i.e., stimuli are 10 seconds apart) produce deep levels of habituation, whereas long ISIs (i.e., 60 seconds apart) produce less habituation (Groves and Thompson 1970; Rankin and

Broster 1992). In all species studied, habituation with high-frequency stimulation leads to more rapid habituation and more rapid spontaneous recovery than does low-frequency stimulation (Rankin and Broster 1992). Rankin and Broster (1992) showed that in *C. elegans*, this sensitivity of spontaneous recovery to frequency of stimulation was true regardless of the number of stimuli delivered (as long as decrement had reached asymptotic levels) and regardless of the level of habituation reached (when levels of habituation were matched between worms habituated with high and low frequencies, rate of recovery was still dependent on frequency of the habituation). This is important for two reasons. The first

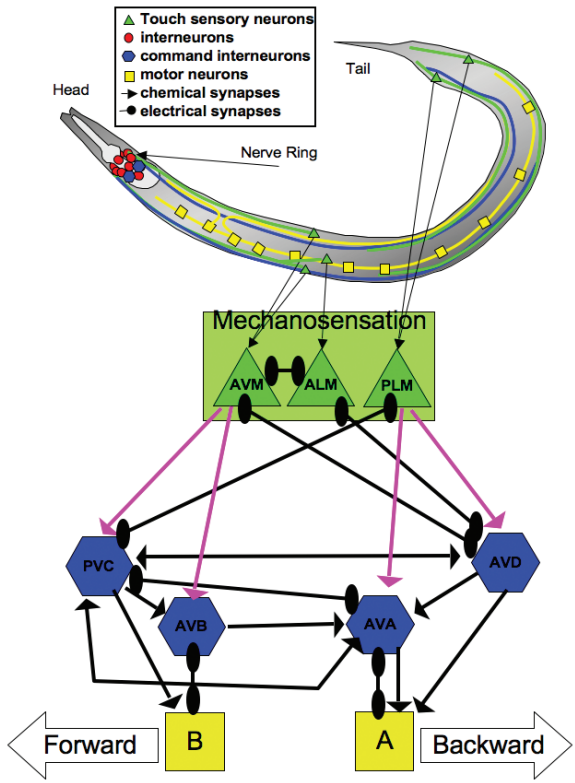

Figure 1. Neural circuit for the response to tap. Cartoon of *C. elegans* showing representatives of the major elements of the neural circuit underlying the response to tap including the five mechanosensory neurons (*green triangles*), two representatives of the four pairs of command interneurons (*blue diamonds*), and a few of the motor neurons regulating forward and backward behavior (*yellow squares*). Lines with arrows represent chemical synapses, and lines ending in ovals represent electrical synapses. The hypothesized sites of plasticity are the chemical synapses between the sensory neurons and the command interneurons (*pink lines*).

is that this difference is the opposite of what would be predicted by fatigue or adaptation, in which the more complete the decrement, the longer the needed recovery. Here, with high frequency, the decrement is rapid and often complete, but recovery is very rapid, whereas with low frequency, the decrement is not complete and yet recovery takes much longer than with high-frequency stimulation. This means that frequency-dependent spontaneous recovery is a second way (in addition to dishabituation) to distinguish whether a behavioral decrement is the result of habituation or the result of sensory adaptation or motor fatigue.

The second reason that frequency-dependent spontaneous recovery is important is that deductions can be drawn from this about mechanisms of habituation. The fact that animals recover rapidly after habituation with high-frequency stimuli (i.e., short interstimulus intervals or ISIs), and more slowly when habituated to the same level to a low-frequency stimulus, led Rankin and Broster (1992) to hypothesize that habituation was not mediated by a single molecular mechanism. Instead, it seems that stimulation at different frequencies likely recruits different cellular mechanisms. Recent unpublished research has provided support for this hypothesis by identifying two genes that differentially affect habituation at short and long ISIs (C. Rankin, unpubl.).

Because running and scoring the behavioral assays for habituation take a great deal of time, a screen for novel genes involved in habituation has been done by only a single lab (Xu et al. 2002), leading to the discovery of one gene, *hab-1*, whose gene product has not yet been identified. More often, the identified candidate gene approach has been used. Because the neural circuit underlying the response to tap has been identified, and because the expression patterns of many thousands of *C. elegans* genes are known, researchers have tested mutations that occur in genes expressed in either the tap sensory neurons or the command interneurons to determine whether these genes have a role in habituation. Thus far, three genes that have a role in habituation have been found using the candidate gene approach: *eat-4*, a homolog of the mammalian glutamate vesicular transporter, VGlut1, expressed on the tap sensory neurons (Lee et al. 1999); *dop-1*, a homolog of the mammalian type-1 dopamine receptor also expressed on the tap sensory neurons; and *cat-2*, a homolog of mammalian tyrosine hydroxylase, the enzyme that makes the neurotransmitter dopamine (investigated after *dop-1* was found to be involved in habituation) (Sanyal et al. 2004). The best studied are *eat-4* mutants, which show normal initial responses to tap but habituate much more rapidly and to a deeper level than wild-type worms at both 10- and 60-second ISIs (Rankin and Wicks 2000).

Although they show slower spontaneous recovery than wild-type worms, their recovery is still frequency-dependent (faster recovery from habituation at a 10-second ISI than from habituation at a 60-second ISI). This ISI-dependent recovery supports the hypothesis that the mutation alters rates of habituation, rather than adaptation or fatigue. This is important because *eat-4* worms do not show dishabituation. The most likely explanation for the more rapid habituation is that without the glutamate vesicular transporter, there are very few filled vesicles and so, with repeated stimulation, the sensory neurons are rapidly depleted. Without the *eat-4* vesicular transporter, restocking of transmitter after depletion is slower than normal; thus, spontaneous recovery is delayed compared to wild-type worms. Together, these findings suggest that although presynaptic release of glutamate from the mechanosensory neurons is important for wild-type short-term habituation, it is not the only mechanism of habituation, as ISI-dependent habituation and spontaneous recovery were both still intact. These data also identify *eat-4* as a gene that is critical for dishabituation.

Both *cat-2* and *dop-1* mutant worms habituate more quickly and deeply than do wild-type worms when habituation is measured by the number of animals reversing to tap, but not when it is measured by reversal length (Sanyal et al. 2004). There are two alternative interpretations of these data: Either dopamine is involved in the regulation of response probability and not response magnitude, or dopamine does not have a role in habituation itself, but may modulate the balance between the head-touch circuit and the tail-touch circuit and not be directly involved in the mechanisms of habituation. *hab-1* was isolated from a screen for worms showing abnormal habituation (Xu et al. 2002). Worms with mutations in *hab-1* show normal responses to tap, habituate more slowly than wild-type worms, and show normal dishabituation. Thus far, the gene product underlying the *hab-1* mutation has not been identified.

C. elegans can show LTM for habituation that can last for up to 5 days (Ebrahimi and Rankin 2006). This is amazing when one considers that this tiny worm only lives about 15–20 days. In *C. elegans,* LTM (tested 24 hours after training) is produced by a spaced or distributed training procedure with four blocks of 20 taps (ISI within blocks was 60 seconds) separated by 1 hour between each training block (Beck and Rankin 1995, 1997; Rose et al. 2002). Long-term memory is not produced by distributed training with a block ISI of 10 seconds or by massed training (all of the stimuli in a row without any rest periods) at either a 60- or 10-second ISI. The inability of shorter ISIs to produce long-term habituation supports the hypothesis that short and long ISIs recruit dif-

ferent cellular mechanisms (Rankin and Broster 1992). The superiority of distributed (spaced) training over massed training appears to be a fundamental feature of long-term memory as it has been shown for different tasks and in many organisms ranging from long-term habituation in *Aplysia* (Carew and Kandel 1973), classical conditioning in *Drosophila* (Tully et al. 1994), to memory for lists of nonsense syllables in humans (Ebbinghaus 1885). In addition to being longer-lived, the memory produced by the spaced-training paradigm is protein-synthesis-dependent. Beck and Rankin (1995) and Rose and Rankin (Rose et al. 2002) examined the effect of protein synthesis inhibition on LTM of habituation in *C. elegans* using heat shock as a gene expression disrupter. Heat shock delivered in the interval between training blocks in the distributed training procedure eliminated the behavioral expression of LTM 24 hours after habituation training.

Although the gene expression cascade resulting in LTM is not known, there is some evidence that glutamate transmission has a role in its induction. The expression of *eat-4* (vesicular glutamate transporter) in the touch sensory neurons first suggested the hypothesis that these cells used glutamate as their neurotransmitter. Because of this, genes that have a role in glutamate neurotransmission were tested for alterations in LTM for habituation. Rose et al. (2003) used the spaced-training procedure and found that worms with mutations in *eat-4* or *glr-1* (a non-NMDA-type glutamate receptor expressed in the command interneurons) do not show LTM. The memory defect of *eat-4*, however, can be rescued by increasing the strength of stimulation. When *eat-4* worms are stimulated with a sufficiently strong stimulus (a train of taps rather than a single tap), they are able to form LTM. This suggests the hypothesis that stronger stimuli cause more glutamate to be released from the sensory neurons (perhaps by mobilizing secondary stores of glutamate), partially overcoming the *eat-4* defect in glutamate transmission leading to LTM. From this, Rose et al. (2003) hypothesized that LTM for habituation to tap is dependent on glutamate release from the mechanosensory neurons activating *glr-1* receptors on the command interneurons.

In mammals, increases or decreases in synaptic strength often are associated with changes in the expression of glutamate receptors (Luscher and Frerking 2001; Malinow and Malenka 2002). To test whether this was the case in worms, Rose et al. (2003) investigated whether LTM training for habituation was accompanied by a change in the expression of GLR-1 using a strain of worms expressing GLR-1 fused to GFP. The amount of GLR-1::GFP expressed by a worm is quantifiable using images taken with a confocal microscope. Indeed, distributed training, which causes long-term

habituation, causes a decrease in expression of GLR-1::GFP compared with expression levels in controls. Because there was no difference in the number of GLR-1::GFP-expressing clusters, Rose et al. (2003) hypothesized that training decreased the number of receptors at each synapse, but did not change the number of synapses. These results suggest that at a cellular level, LTM for habituation is mediated by a down-regulation of GLR-1 expression. Together, these studies suggest an important role for glutamate neurotransmission and, more specifically, glutamate receptor trafficking, in the formation of LTM of habituation of the tap-withdrawal response. This mechanism is remarkably similar to the mechanisms described for some forms of long-term depression in mammals.

Habituation is traditionally considered to be a nonassociative form of learning, but it can be involved in associative learning tasks. Rankin (2000) demonstrated this for *C. elagans* using context conditioning with habituation. In context conditioning, cues from the environment may have a role in memory retrieval (Wagner 1976); the context serves as the conditioned stimulus (CS) and the experience (i.e., habituation to tap) as an unconditioned stimulus (US). To test for context conditioning, *C. elegans* worms were trained under one set of environmental conditions and then tested 1 hour later under either the same or different conditions. The context cue used was the presence or absence of a distinctive taste, sodium acetate, on the surface of the agar. Worms were trained with 30 taps on either plain agar plates or sodium-acetate-treated plates, moved to plain plates for 1 hour, and then rehabituated on either plain or sodium acetate plates. Those worms that were both trained and tested on sodium acetate showed greater retention of the earlier training than did worms that were trained and tested on different types of plates. This conditioning is lost if worms are preexposed to the sodium acetate prior to training (latent inhibition) or if they are exposed to sodium acetate for the hour between training and testing (extinction). The results of these experiments demonstrate that worms are capable of associative learning in the form of context conditioning.

Although this review has focused on habituation in the mechanosensory system, other researchers have made progress in understanding a number of other learning paradigms in *C. elegans*. Several assays have been developed that involve chemosensory learning. A differential classical conditioning paradigm was developed that consisted of preexposing worms to two odors and pairing one odor (conditioned stimulus, CS+) with and the other odor (CS–) without food. After training, worms significantly preferred the odor that had been paired with food (Wen et al. 1997). Differential classical conditioning also can be demonstrated by pairing an

aversive stimulus, such as garlic, with the CS+ odor. In this paradigm, worms significantly prefer the CS– odor that was not paired with the aversive stimulus. Since the development of these chemosensory paradigms, several others have been developed, including pairing odors and/or tastes with feeding states (fed vs. starved; Saeki et al. 2001; Ishihara et al. 2002). Several genes have been identified with genetic screens for mutants with defective differential classical conditioning in which specific chemosensory stimuli are paired with food or no food. *lrn-1* and *lrn-2*, which have not yet been identified molecularly, show normal chemotaxis and chemotactic habituation but are deficient for associative conditioning (Wen et al. 1997; Morrison et al. 1999). The glutamate receptor subunit, GLR-1, important for LTM for habituation to mechanosensory stimulus, has also been shown to be important for chemosensory learning. *glr-1* mutants show deficiencies for both chemotactic habituation and differential classical conditioning (Morrison and van der Kooy 2001). Finally, mutations in the gene *hen-1*, whose gene product is an LDL receptor-like secretory protein, lead to abnormal sensory integration of chemosensory signals and do not exhibit learning (Ishihara et al. 2002).

Behavioral plasticity has also been shown in *C. elegans* for thermotactic behavior (Hedgecock and Russell 1975). Worms can learn to associate a particular temperature with the presence or absence of food. This can be measured by placing the worms on a temperature gradient; if the worms have recently been well fed in an environment at a constant temperature, they will migrate to this temperature when placed on the gradient. Conversely, if the worms have recently experienced starvation at a constant temperature, they will avoid this temperature when placed on the gradient. This behavior is termed isothermal tracking. The neural circuit underlying this behavior has been determined, and a number of genes involved in this learning task have been identified (Mori and Ohshima 1995). The neurotransmitter serotonin is hypothesized to mimic a well-fed state in *C. elegans;* exogenous application of serotonin to starved worms during conditioning to a temperature mimics the effect of a food-rich environment. The neurotransmitter octopamine is thought to mimic a starved state in *C. elegans;* exogenous octopamine applied to well-fed worms during cultivation causes a subsequent avoidance of the cultivation temperature (Mohri et al. 2005).

To investigate mutants defective for forming associations between temperature and food, a genetic screen was done for worms abnormal for updating their isothermal tracking. From this, three mutants, *aho-1, aho-2,* and *aho-3,* were identified (Mohri et al. 2005). These mutants show normal thermotaxis when they are cultivated in a well-fed state, but

they cannot learn to avoid the cultivation temperature if they are conditioned in a starved state. This screen illustrates that there are two separable aspects of thermotaxis: the thermal memory and the associative learning. Several other genes implicated in thermotaxis have also been identified. Neuronal calcium sensor-1 (*NCS-1*) is one such gene; *ncs-1* mutants always move toward colder temperature in the thermotactic learning assay (Gomez et al. 2001). In addition, *HEN-1* (shown to have a role in chemosensory learning) is involved in thermotaxis learning.

Despite a relatively small nervous system and brief life span, *C. elegans* are equipped with a high degree of plasticity. *C. elegans* come prewired to learn about their environment and to use the memories of their past experience to guide their future behavior. Because the behavioral rules of simple forms of learning such as habituation and classical conditioning appear to be universal, it is likely that mechanisms underlying these behaviors in *C. elegans* or *Drosophila* will turn out to be highly conserved across evolution. The simplicity of the worm nervous system, the complete connectivity map, the genomic information, and the genetic strengths of *C. elegans* will continue to provide an ideal model for dissection of learning and memory. *D. melanogaster,* on the other hand, has offered a different set of advantages for dissection of memory formation: consolidation and its retrieval.

PAVLOVIAN LEARNING IN *DROSOPHILA*

The Paradigm

One of the most robust and highly studied memory paradigms in *Drosophila* is a Pavlovian assay in which the animals learn to associate a pure chemical odor (CS) with either an electric shock punishment or a sugar reward (Tempel et al. 1983; Tully and Quinn 1985). The aversive version of this assay, which evolved from an earlier operant olfactory learning assay (Quinn et al. 1974), has for several reasons become the most practical for forward mutagenesis screens and for genetic dissection of memory consolidation. We have chosen to focus discussion on this Pavlovian assay, but it should be noted that impressive progress has been made with several different learning paradigms, including operant and Pavlovian visual learning (Heisenberg et al. 2001), an operant spatial learning procedure (Wustmann and Heisenberg 1997), and assays of courtship conditioning (Siwicki and Ladewski 2003; Mehren et al. 2004). Many of the findings with this olfactory task in *Drosophila* also mirror those with olfactory associative tasks in other insects such as the honeybee *(Apis mellifera*) (Menzel and Muller 1996; Menzel 2001).

Many of the properties of Pavlovian learning first described in other species also are observed in flies, including order dependence, temporal specificity, conditioned inhibition, conditioned excitation, CS and US preexposure effects, and extinction (Tully and Quinn 1985; Dubnau 2003). An advantage of the Pavlovian olfactory paradigm is that successful performance only requires that animals perceive the odors and the electric shock and have the ability to run away from the shock. So sensorimotor defects can only cause a performance defect in this assay if they perturb sensation or avoidance of either of the two odors or the electric shock used in the assay. These task-relevant sensorimotor responses can be measured directly to reasonably rule this out.

A second advantage of this assay is that a single round of odor shock training yields asymptotically high levels of learning in wild-type flies. This has proven to be valuable because even subtle defects can be observed, but it also has turned out to be key for investigation of memory consolidation. In most memory tasks, additional training is required for induction of LTM (discussed below in detail). Often, however, additional training also yields higher levels of acquisition (e.g., in water maze learning in rodents). As a result, manipulations that disrupt LTM, which requires multiple training sessions, can do so either by a direct impact on consolidation or by decreasing the rate of acquisition over multiple trials. This confound is minimized in this case by the rapid formation of maximal learning with a single training session.

For these reasons, a majority of the mutants with memory defects in flies have been identified using this Pavlovian olfactory paradigm (Waddell and Quinn 2001; Margulies et al. 2005), although a number have subsequently been found to also disrupt other learning tasks (Duerr and Quinn 1982; Rees and Spatz 1989; Engel and Wu 1996; Wustmann et al. 1996; O'Dell et al. 1999; Cho et al. 2004), indicating that cellular mechanisms may be shared across modalities and tasks.

The Early Genetic Screens

To date, three forward mutagenesis screens—two "reverse genetic" screens, and a good deal of reverse genetic hypothesis testing—have yielded a long list of genes implicated in olfactory memory. The first two mutants to be identified molecularly turned out to be a phosphodiesterase (*dunce*) (Byers et al. 1981; Chen et al. 1986) and an adenylyl cyclase (*rutabaga*) (Livingstone et al. 1984; Levin et al. 1992), indicating a remarkable convergence on the cAMP pathway, which also had been implicated in memory and synaptic plasticity from work in *Aplysia* (Kan-

del and Schwartz 1982). The identification of a role for cAMP signaling also is entirely consistent with results from pharmacological approaches to dissect mechanisms underlying classical (olfactory) conditioning of the proboscis extension reflex in honeybees (Menzel and Muller 1996; Menzel 2001). Although this first screen, conducted by Benzer's group and continued by Quinn's group, was small in scale compared with the saturation mutagenesis screens that were being performed for development, the early molecular identification of just two mutants, *dunce* and *rutabaga*, provided an entry point into both the biochemical signaling pathways and the anatomical circuitry of memory. The discovery of these two genes has generated three decades of scientific momentum that continues to this day.

Reverse genetic hypothesis testing established the involvement in memory of additional components of the cAMP cascade, including a stimulatory G protein (Connolly et al. 1996), catalytic and regulatory subunits of protein kinase A (Drain et al. 1991; Skoulakis et al. 1993; Li et al. 1996; Goodwin et al. 1997), the neurofibromatosis-1 (NF-1) protein (Guo et al. 1997), and the cAMP-responsive transcription factor CREB (Yin et al. 1994, 1995; but see also Perazzona et al. 2004 and discussion below). The *amnesiac* gene also may be a component of this biochemical pathway because it encodes an open reading frame that is predicted to give rise to several neuropeptides, one of which has weak homology with the pituitary adenylyl-cyclase-activating peptide (PACAP) (Feany and Quinn 1995; DeZazzo et al. 1999).

In addition to yielding insight into a biochemical signaling pathway involved in memory, identification of *dunce* and *rutabaga* also provided an entry into the anatomical circuitry of memory. Both proteins show elevated expression in a part of the brain called the mushroom bodies (MBs) (see Fig. 2) (Nighorn et al. 1991; Han et al. 1996). MBs already had been implicated in an association center by work in several different insect species (Hammer and Menzel 1998; Strausfeld et al. 1998; Heisenberg 2003; Davis 2005), and in *Drosophila,* it was known that mutants with structural defects in MB anatomy exhibited deficient olfactory learning (Heisenberg et al. 1985; for recent reviews, see Gerber et al. 2004b; Davis 2005; Margulies et al. 2005). Together with the expression pattern of *dunce* and *rutabaga*, this prompted the Davis lab to conduct an enhancer-trap screen for transposon insertion alleles that yielded reporter expression in MBs (Han et al. 1996). These authors went on to demonstrate the roles of several of these genes in memory formation (Skoulakis and Davis 1996; Grotewiel et al. 1998; Cheng et al. 2001). With this approach, they identified additional components of the cAMP pathway

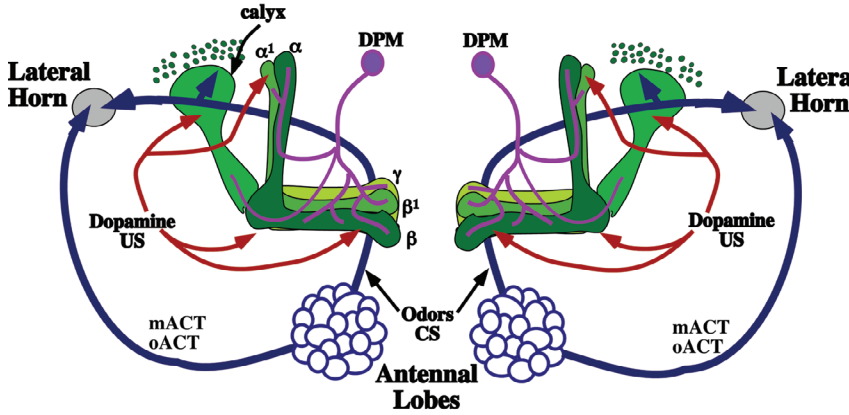

Figure 2. Olfactory memory circuit in *Drosophila*. Anterior view of the neural circuitry involved in olfactory associative memory. The mushroom body (MB) is believed to be a site where CS (odors) and US (electric shock) are associated. The primary olfactory processing center is the antennal lobe (*blue*). The CS (odor) is conveyed out of antennal lobe to lateral horn (*gray*) and MB (*green*) by several different projection neuron tracts, the mACT, oACT, and iACT (*blue arrows*). The US is believed to reach the MB via dopaminergic inputs to calyx and lobes (*red arrows*). Neuromodulation by dorsal-paired-medial neurons (DPM; *pink*) is required after training for memory consolidation. The calyx contains the dendritic field of the MB. MB axon terminals are contained in the lobes. MBs consist of three types of Kenyon cells: α, β neurons, whose axonal projections comprise the α and β lobes (*dark green*); α′ β′ neurons, whose projections enter the α′ β′ lobes (*green*); and γ neurons, whose projections form the γ lobes (*light green*).

as well as several genes with no known connection to the cAMP pathway, including 14-3-3, a signaling molecule (Skoulakis and Davis 1996); *volado* (Grotewiel et al. 1998), an integrin; and *fasII* (Cheng et al. 2001), an adhesion molecule. Using the same approach, Preat's group also identified *crammer* (Comas et al. 2004), a fly cystein proteinase in the cathepsin family, although of note is their finding that *crammer* may actually function in glial cells, not in MBs. Thus, this second round of screening suggested more complex biochemical signaling underlying memory and provided additional evidence that MBs participate in memory.

The direct demonstration that MB neurons are part of the memory circuit came from interventionist experiments to disrupt MB function. There now is a wealth of evidence that MBs participate in olfactory memory (for review, see Gerber et al. 2004b; Davis 2005; Margulies et al. 2005) (see below), and a simple model of olfactory associative learning in flies has been proposed in which a single signaling pathway (cAMP) and a

single neural structure (MBs) explain memory formation and storage. In this simplified model, olfactory and neuromodulatory inputs to MB Kenyon cells would induce cAMP-dependent synaptic plasticity. Information storage would be entirely contained in MBs, and memory consolidation would occur therein. This model has tremendous intuitive appeal and is certainly an important part of the story, but findings from three levels of analysis (dissection of memory phases, additional gene discovery, and investigations of the relevant neuroanatomy) suggest the involvement of both more complex cellular machinery and a more complex neural circuitry.

Dissection of Memory Phases

One of the defining features of memory is a gradual consolidation from a labile state that can be easily disrupted into progressively more stable and lasting forms. Each of these so-called memory phases can be distinguished with experimental interventions such as behavioral manipulation, pharmacological inhibition, genetic disruption, or anatomical lesions. In flies, the combined use of these approaches has succeeded to dissect memory after olfactory memory into multiple mechanistically distinct memory phases (for a detailed discussion, see Margulies et al. 2005).

Long-lived memory, for example, appears to consist of at least two different cellular mechanisms. First, LTM is sensitive to pharmacological inhibition of protein synthesis (Tully et al. 1994) as well as to genetic perturbation of the cAMP-responsive transcription factor, CREB (Yin et al. 1994, 1995). In contrast, anesthesia-resistant memory (ARM), which is operationally defined as being resistant to cold-shock anesthesia, is disrupted in *radish* mutant animals (Folkers et al. 1993). Unlike LTM, ARM is resistant to pharmacological inhibition of protein synthesis as well as to genetic disruption of CREB (Tully et al. 1994). In addition, unlike ARM, LTM is normal in *radish* mutant animals. Further evidence that these two forms of memory are genetically distinct comes from more recent findings that *nalyot* (DeZazzo et al. 2000), *Notch* (Ge et al. 2004; Presente et al. 2004), *crammer* (Comas et al. 2004), *nebula* (Chang et al. 2003), *tequila* (Didelot et al. 2006), and NMDAR1 (Xia et al. 2005) mutations each disrupt LTM and leave *radish*-dependent ARM intact.

These two memory phases also have differing behavioral characteristics (Tully et al. 1994). CREB-dependent LTM, for instance, does not appear for at least several hours after training and is not induced unless the animals are subjected to multiple training sessions spaced out over time (typically, ten cycles of training with a 10-minute rest interval

between each). In contrast, *radish*-dependent ARM appears more rapidly (less than 1 hour) even after one training session, but it is more robust and longer lived after multiple training sessions, either massed together or spaced out over time. These two mechanistically distinct memory phases nevertheless can coexist temporally in animals that are given spaced training, which induces both forms of memory. After spaced repetitive training, ARM can last for up to several days and LTM can last for 1 week.

Finally, induction of ARM and LTM appears to be gated by distinct regulatory mechanisms. Induced expression of the Mζ isoform of protein kinase C appears to rescue memory of the *radish* mutant (and can enhance memory in otherwise normal animals), suggesting the possibility that activation of this kinase is sufficient to signal the induction of ARM (Drier et al. 2002). The *radish* gene itself was originally reported to encode a PLA2 based on failure to complement a PLA2 transposon allele (Chiang et al. 2004). This conclusion appears to be incorrect, however, because the noncomplementation cannot be reproduced (A. Blum and J. Dubnau, unpubl.). More recently, Folkers et al. (2006) identified the molecular lesion in *radish*. Their findings indicate that *radish* encodes a previously unknown protein with potential phosphorylation sites for PKA. Transgenic expression of this protein is capable of rescuing the original *radish* allele. Thus, to date, protein kinase C signaling and possibly PKA signaling via phosphorylation of *radish* are implicated in ARM. Formation of LTM, on the other hand, is gated by the CREB transcription factor. Induced overexpression of a CREB-blocking isoform is sufficient to prevent LTM formation even after spaced training (Yin et al. 1994). In contrast, overexpression of a CREB-activating isoform has been reported to enhance LTM, leading to its induction after even a single training session (Yin et al. 1995). Perazzona et al. (2004) were not able to reproduce this last finding and identified a point mutation in the activator transgene, which called the enhancement observed by Yin et al. into question. However, Perazzona et al. also observed a leaky disruption of LTM with the CREB blocker isoform even prior to induction. This leaky effect has not been seen by other groups, which raises the possibility that the discrepancy with the CREB activator could be due to methodological differences. This seems likely, given the large body of evidence supporting a role for CREB in LTM in numerous model systems. Nevertheless, the study by Perazzona et al. raises serious questions about the mechanisms by which the CREB activator was able to enhance memory given the presence of a mutation in the transgene (for a detailed discussion, see Margulies et al. 2005).

Taken together, the above behavioral, pharmacological, and genetic experiments indicate that in *Drosophila*, ARM and LTM are mechanistically distinct forms of long-lived memory. It is not yet known whether this dissection will turn out to be conserved across phyla because the genetic reagents have not been available to test this possibility. It is worth mention, however, that an intermediate phase of memory and of cellular plasticity has been described in *Aplysia* (Sutton and Carew 2000; Sutton et al. 2001). This memory phase requires persistent activation of PKA, and like ARM, it appears more quickly than LTM, does not last as long, and is independent of transcription. Unlike ARM in *Drosophila*, however, this intermediate phase of memory in *Aplysia* is blocked by inhibitors of translation.

Like long-lived memory, earlier memory can also be genetically and behaviorally distinguished. During the first few hours after a single training session, for example, at least two distinct memory mechanisms are at play: *radish*-dependent ARM gradually appears during the first hour after training, and *amnesiac*-dependent anesthesia-sensitive middle-term memory (MTM) gradually fades (Tully and Quinn 1985; Folkers et al. 1993; Tully et al. 1996). Again, these two forms of memory coexist for several hours. In addition, memory prior to formation of ARM also appears to rely on more than one mechanism. This is revealed by comparing the memory decay kinetics in wild-type and several single-gene mutant animals (for a detailed discussion, see Dubnau and Tully 1998). Mutations in *dunce, rutabaga,* and NF-1 (Tully and Quinn 1985; Guo et al. 2000), for example, have lower than normal memory measured immediately after training, but they also appear to rapidly lose what memory they do form. These mutations have therefore been thought to disrupt primarily STM. In contrast, mutations in *linotte* (Dura et al. 1993, 1995; Bolwig et al. 1995; Moreau-Fauvarque et al. 2002), *latheo* (Boynton and Tully 1992), *volado* (Grotewiel et al. 1998), 14-3-3 (Skoulakis and Davis 1996), *fas II* (Cheng et al. 2001), or S6KII (Putz et al. 2004) cause deficient performance immediately after training, but they exhibit normal rates of memory decay thereafter. These mutations are thought to primarily disrupt acquisition. To add to this complexity, it should be noted that no single-gene mutation completely abolishes learning. Null alleles of *dunce* or *rutabaga*, for instance, still have about 50% residual learning measured 2 minutes after training. Thus, the potential exists for as yet undiscovered cellular mechanisms of memory storage. Together, the phenotypic analysis of single-gene mutants indicates that multiple cellular mechanisms subserve memory storage and processing.

Additional Gene Discovery

The early "vegetable mutants" were identified via an ethylmethane sulfonate (EMS)-mediated chemical mutagenesis focused on the X chromosome (named after vegetables because they were supposedly as clever). This screen served as a proof of principle that the "single-gene approach" could greatly enhance our understanding of memory, and it set in motion a series of reverse genetic hypothesis-testing ventures such as gene disruptions of additional components of the cAMP signaling cascade and identification of additional genes with elevated expression in MBs (discussed above). As a follow-up to this early screen, Tully's group conducted two transposon-based behavioral screens focusing on the mutations on the autosomes. Like the earlier screens, these so-called "dog screens" (the mutants were named after Pavlov's dogs) are yielding insight into both the biochemical pathways and anatomical circuits of olfactory memory. The first, a small-scale pilot screen, led to the identification of *nalyot* (DeZazzo et al. 2000), *latheo* (Boynton and Tully 1992), and *linotte* (Bolwig et al. 1995), which were discussed above.

Interestingly, each of these mutants identifies genes that have no obvious connection to the cAMP pathway, consistent with the involvement of additional biochemical "circuitry." *nalyot*, which is an allele of the adf-1 transcription factor (DeZazzo et al. 2000), has both a subtle learning defect and a profound deficit in LTM. *nalyot* mutants also exhibit defective synaptic growth at the NMJ. *latheo* encodes a subunit of the origin recognition complex involved in DNA replication and yet it has a role in neuronal proliferation (Pinto et al. 1999). Although at face value this suggests a developmental etiology of the mutant phenotype, the *latheo* protein also is expressed at presynaptic boutons at the NMJ, where it has been reported to have a role in synaptic physiology (Rohrbough et al. 1999). Finally, the *linotte* mutation is caused by a transposon insertion between the *derailed* receptor tyrosine kinase and a novel protein. There have been conflicting claims in the literature (Dura et al. 1993, 1995; Bolwig et al. 1995; Moreau-Fauvarque et al. 2002), but the balance of evidence supports the conclusion that the memory defect is due to a disruption of the *derailed* open reading frame (for discussion, see Margulies et al. 2005).

This second-generation transposon mutagenesis screen, like the earlier EMS screen, illustrated the potential of unbiased forward mutagenesis for identification of cellular mechanisms of learning. Like the earlier screen, however, this transposon screen was small in scale and focused on memory after a single training session without activating LTM. To iden-

tify genetic components of the cascade of CREB-dependent genes responsible for LTM, Dubnau et al. (2003b) used two complementary approaches. First, a microarray screen was used to identify 42 transcripts whose levels are significantly changed after spaced versus massed training. The rationale of this approach was to bias toward transcripts acutely involved in formation of CREB-dependent LTM. Second, a large-scale behavioral screen was conducted to identify transposon insertion alleles ("the dog alleles") with defective 1-day memory after spaced training. In total, this behavioral screen identified 60 alleles, defining 57 loci. The memory defects of these mutants could derive directly from a disruption of CREB-dependent LTM or indirectly from a defect in an earlier phase of memory. Because the transposons used in this screen are of the enhancer trap design, they also have facilitated rapid investigation of reporter expression pattern for each locus. In this way, "the dog alleles" provide entry points to investigate both cellular machinery and anatomical circuitry.

The *pumilio* translational repressor was identified in both the microarray and the behavioral screen. First identified in screens for defects in embryonic development (Macdonald 1992), *pumilio* is part of a well-studied pathway involved in local translational repression in the early embryo. In addition to *pumilio*, other components of this group of genes also were identified in either the behavioral screen or the expression screen. From the microarray screen, these include *staufen*—a protein with a known role in mRNA localization, orb—the fly homolog of cytoplasmic polyadenylation-element-binding protein, *moesin*, and eIF2G. From the behavioral screen, *oskar* and eIF5C were identified.

Direct evidence that this pathway participates acutely in memory formation derived from use of a temperature-sensitive allele of *staufen* (Dubnau et al. 2003b). Using temperature-shift experiments, it was possible to demonstrate that *staufen* function is required after training, during the memory consolidation period. These findings suggest the hypothesis that mRNA localization and translational control have an acute role in LTM formation, perhaps via a mechanism involving translation of mRNAs present at or near relevant synapses. Moreover, the transcriptional induction of several of these genes indicates that part of the CREB-mediated gene expression cascade includes a wholesale up-regulation of the machinery for mRNA regulation. It remains to be determined which transcripts are the relevant translational targets for memory, although a recent study (Ashraf et al. 2006) suggests that calcium/calmodulin-dependent protein kinase II (CaMKII) may be one because its translation in antennal lobe (AL) appears to be directly stim-

ulated by spaced training. Moreover, pan-neuronal perturbation of CaMKII function via expression of an RNA interference (RNAi) transgene appears to inhibit LTM.

In addition to unbiased screens, some of the most informative gene discovery efforts have continued to derive from hypothesis testing. In some cases, candidate genes have been selected based on modeling of human disease (humans as a model system for fly memory?). *neurofibromatosis-1* (*NF-1*), for example, is associated with cognitive disorders in humans and appears to have an acute role in learning in flies and in LTM in rodents (Silva et al. 1997; Guo et al. 2000; Costa et al. 2001). Interestingly, the NF-1 protein provides a potential link between cAMP and *ras*-mediated signaling pathways because the NF-1 protein, which contains a GTPase-activating protein (GAP)-related domain (GRD), inhibits *ras* activity and also regulates adenylyl cyclase activity (Guo et al. 1997; The et al. 1997; Tong et al. 2002; Hannan et al. 2006).

A second example of a human disorder suggesting a candidate gene approach for fly learning comes from Down's syndrome. A candidate gene from the Down's syndrome trisomic region, Down's syndrome critical region 1 (DSCR1), is a calcineurin inhibitor protein in the calcipressin family. *nebula*, the fly homolog of human DSCR1, has been shown to have a role both in learning and in LTM formation (Chang et al. 2003). Either loss of function or overexpression in MBs of the *nebula*+ cDNA results in defective LTM, suggesting that the levels of *nebula* function are under tight control. Manipulation of *nebula* expression also is associated both with altered levels of CREB phosphorylation and with altered calcineurin activity.

The transmembrane receptor *Notch* also has been shown to have a role in LTM formation (Ge et al. 2004; Presente et al. 2004). Following a hunch, two groups independently investigated the role of *Notch* signaling, each making use of a different temperature-sensitive allele. Although learning appears not to be affected by disruption of *Notch*, LTM can be blocked with the temperature-sensitive alleles or by spatially restricted inhibition of *Notch* in MB neurons (Ge et al. 2004; Presente et al. 2004). Remarkably, induced overexpression of *Notch* is sufficient to enhance memory, leading to LTM formation after only one training session (Ge et al. 2004).

Taken together, the parallel use of forward mutagenesis, microarray screens, identification of genes with expression in MBs, and hypothesis-driven candidate gene selection has produced a staggering list of genes with roles in olfactory memory. As a general rule, genetic manipulation of these genes yields relatively phase-specific impacts on memory. The

challenge, however, is to use these reagents to uncover the mechanisms by which information is processed during memory consolidation through these various phases. Part of the problem is that in most cases, we do not know which genes interact in a biochemical sense within the same cells and which function in different cells and "interact" via neural circuit properties. Put another way, two possible hypotheses present themselves. First, mechanistically distinct memory phases could reflect overlaid biochemical pathways acting within the same set of neurons (i.e., MB Kenyon cells), but each with different kinetics and pharmacological susceptibilities. On the other hand, some or all of the complexity of memory consolidation could involve a systems-level processing of information within a larger neural network. In this case, gene interaction need not be occurring only in a biochemical sense. Instead, each memory phase could reflect the use of different cellular mechanisms in distinct parts of a broader neural circuit.

Neural Circuitry of Olfactory Memory

The *Drosphila* brain, like that of other insects, consists of highly intricate brain structures. The MB is a highly conserved and complex neuropil structure that receives multimodal inputs, including major olfactory inputs from the antennal lobes (AL). Work of classical anatomists (see, e.g., Strausfeld 1976; Strausfeld et al. 1998) as well as some elegant electrophysiological and pharmacological experiments in insects with larger and more accessible brains initially suggested the MB as a structure involved with forming multimodal associations (Gronenberg 1987; Laurent and Naraghi 1994; Hammer and Menzel 1998; Rybak and Menzel 1998; Lozano et al. 2001). The tools available in *Drosophila* have permitted the type of interventionist approaches needed to test this hypothesis in some mechanistic detail. The availability of the Gal4 expression system has been key in this endeavor. This bipartite expression system consists of two panels of strains. The first are called "driver lines" that express the yeast Gal4 transcription factor in a reproducible subset of neurons under control of enhancers from endogenous fly genes (these are identified fortuitously in large-scale screens). The second consists of reporter genes under control of the Gal4-responsive promoter (UAS). More recently, several methods of further controlling the temporal induction of these lines have been established (Mao et al. 2004; McGuire et al. 2004).

The evidence now is overwhelming that *Drosophila* MBs are a key site of CS–US association, at least for olfactory memory (Heisenberg et al. 1985; de Belle and Heisenberg 1994; Connolly et al. 1996; Dubnau et al.

2001; McGuire et al. 2001; Schwaerzel et al. 2002). One of the more informative findings is that transgenic expression in MBs of the *rutabaga* adenylyl cyclase is sufficient to restore normal levels of memory to *rutabaga* mutants, at least out to 3 hours (Zars et al. 2000a,b). This latter finding also has been demonstrated with induced expression in adult MBs of the *rutabaga*+ cDNA (Mao et al. 2004; McGuire et al. 2004). These findings support the conclusion that cAMP-dependent synaptic plasticity may only be required in MBs, at least for early memory.

Further information about the role of MBs has derived from a now standard method for reversibly silencing neural activity. This method makes use of a Gal4-responsive transgene expressing a temperature-sensitive and dominant-negative *shibire* cDNA (Kitamoto 2001, 2002). *Shibire*, the fly homolog of dynamin, is required for vesicle endocytosis, a rate-limiting step in neurotransmitter vesicle recycling. When combined with a Gal4 driver, this *shibire* transgene allows the reversible silencing in a defined population of neurons of dynamin-dependent function, which mainly affects small-molecule synaptic transmission.

Transient inhibition of neurotransmission in MB Kenyon cells is sufficient to block memory retrieval after either appetitive or aversive olfactory conditioning (Dubnau et al. 2001; McGuire et al. 2001; Schwaerzel et al. 2002, 2003). In contrast, normal acquisition occurs even while MB output is blocked. These results, together with the evidence mentioned above, have led to a model in which both the CS and US are received during learning by MB Kenyon cells. CS–US association then requires cAMP-dependent coincidence detection in MB Kenyon cells, and synaptic plasticity therein is hypothesized to support the behavioral association. In this model, output from the MB only is required during memory retrieval because the relevant synaptic plasticity is driven by inputs only. Both CS and US inputs, on the other hand, would be required during training. Here too, the *shibire* approach has provided support for this model. Transient inhibition of a population of projection neurons (PNs), which convey olfactory information from AL to MB calyx as well as to the lateral horn (LH), is sufficient to block acquisition (Schwaerzel et al. 2002). Again, this supports the hypothesis that CS inputs to MBs are an important part of the neural circuitry. There is also evidence supporting the notion that US inputs to MBs are required. Again using the *shibire* approach, Schwaerzel et al. (2003) provided a reasonable argument that dopaminergic inputs to MBs convey the US for electric shock learning. Interestingly, appetitive conditioning was not affected by this disruption of dopaminergic transmission, but instead, it appeared to depend on release of octopamine (an invertebrate counterpart of norepinephrine).

This dissection of the reinforcing roles of dopamine and octopamine gained further support from a more recent study of learning in the larva (Schroll et al. 2006). This study used transgenic expression of channelrhodopsin-2, a light-activated channel, to directly stimulate dopaminergic or octopaminergic neurons using flashes of light. Remarkably, they found that they could substitute a light flash for the US stimulus. Activation of dopaminergic neurons substituted for an aversive stimulus, and activation of octopaminergic population of neurons substituted for an appetitive stimulus. Together, these findings raise evolutionary questions about the psychology of reward and punishment because in vertebrates, dopaminergic signaling is thought to have a role in reinforcing reward. This apparent switch in the role of dopamine between vertebrate and invertebrate animals, however, may not sufficiently reflect the complex role of these neurotransmitters in modulating behavior. For example, dopamine in flies, as in vertebrates, appears to modulate arousal (Andretic et al. 2005).

Nevertheless, the above studies support the idea that neuromodulatory input to MBs has a role in mediating the US. These findings thus are also consistent with the above model in which coincidence detection in MBs underlies CS–US associations for both appetitive and aversive conditioning. Several recent studies indicate, however, that the circuit underlying olfactory memory may be substantially more complex.

MB Kenyon cells, for instance, consist of several developmentally distinct subtypes, each of which has axon terminals in different so-called lobed structures of the MB (Fig. 2) (for a recent review, seeMargulies et al. 2005). These may also reflect functionally distinct subsets of the MB. Using *rutabaga* transgenic rescue as an assay, Zars et al. (2000a,b) were able to provide a clue about functional subdivisions within MBs. They used a panel of Gal4 lines to drive expression of the *rutabaga*+ cDNA in an otherwise *rutabaga* mutant background. Each Gal4 line yielded expression in varying subsets of MB Kenyon cells. The Gal4 lines that were able to rescue the *rutabaga* STM defect (after a single aversive training session) always showed some reporter expression in γ lobes; two Gal4 lines showed no evidence of rescue and did not express in γ lobes. Together, these results support the hypothesis that STM requires *rutabaga* function only in γ lobes. Two caveats should be kept in mind, however. First, the two Gal4 lines that only express outside of γ lobes, and which did not show rescue, also show very low levels of transgenic expression. It is therefore difficult to rule out the possibility that the levels of *rutabaga*+ expression were insufficient in these cases. Second, the two Gal4 lines whose expression appears entirely restricted to γ lobes also

showed only partial rescue, compared with the full rescue seen in pan MB-expressing lines. Thus, the possibility remains that STM may also require *rutabaga* function more generally in MB Kenyon cells. Some recent evidence for this comes from a report that dorsal paired medial (DPM) cell contacts with α′/β′ lobes may be important for normal 1-hour memory (Keene et al. 2006). This apparent contradiction with the findings from *rutabaga* rescue in γ lobes also may reflect differences in which memory phase is being manipulated, but this remains to be tested.

There also is accumulating evidence that the circuitry required to form and to store olfactory memories goes beyond MBs and the CS and US inputs. The strongest evidence comes from the discovery that the *amnesiac* gene is expressed and functionally required not in MB neurons, but instead in DPM neurons (Waddell et al. 2000). These large, presumably neuropeptide-releasing neurons appear to project primarily to the MB lobes (Ito et al. 1998; Waddell et al. 2000). Indeed, the terminals of these beautiful neurons decorate all of the MB lobes, although its contacts with α′/β′ lobes may be sufficient for 60-minute memory (Keene et al. 2006). Transient inhibition of dynamin-dependent neurotransmitter release from these neurons (via the *shibire* approach) reveals that DPM cell function is required during the storage of memory out to at least a 3-hour time point (again, both for appetitive and aversive conditioning) (Waddell et al. 2000; Dubnau et al. 2003a; Keene et al. 2004; Yu et al. 2005). In contrast, DPM cell function appears to be dispensable both during the training procedure and during retrieval. Importantly, normal 2-minute memory can be formed, stored, and retrieved even while DPM cell function is blocked. Taken together, these data strongly support the hypothesis that MTM maintenance requires a neuromodulatory role of DPM neurons. This notion also is largely consistent with the behavioral defect in *amnesiac* animals, which exhibit relatively normal learning but are defective in MTM (Quinn et al. 1979; Tully and Quinn 1985; Feany and Quinn 1995; DeZazzo et al. 1999). A word of caution, however, is that *amnesiac* is thought to encode a neuropeptide, whose release would not likely be blocked by *shibire*. The behavioral effects seen with *shibire* are thus likely due to inhibition of another neurotransmitter, probably acetylcholine (Keene et al. 2004). Thus, there is a conceptual disconnect between the role of *amnesiac*-encoded protein and the DPM neurons in which they are expressed. Nevertheless, these studies point to an active processing of information underlying MTM that presumably requires neural activity after the training event is complete. DPM neurons may be modulating activity in MBs, but they still point to a broader circuit and more dynamic information processing than was previously thought.

In the case of LTM, virtually nothing is known about the circuitry involved in either storage or retrieval (for a detailed discussion, see Margulies et al. 2005). As was true for early memory, the site of expression and function of the relevant genes may shed some light. In the case of *Notch*, *tequila,* and *nebula*, expression in MBs appears to have a role (Chang et al. 2003; Presente et al. 2004; Didelot et al. 2006). But, on the other hand, reporter expression of several mutants with defective LTM yields expression only outside of MBs (Dubnau et al. 2003b). For most of the genes involved in LTM, the anatomical site of action has not been investigated, and functional manipulation of the circuitry in which these genes are expressed has not yet been accomplished. Nevertheless, the available data indicate that even early memory involves substantially complex neural circuitry. The maintenance/consolidation of memory during the first few hours requires ongoing processing in a circuit that certainly includes, but is not limited to, MBs. This notion is further supported by several recent studies using functional imaging of experience-dependent neural activity in living animals (Wang et al. 2004; Yu et al. 2004, 2005; Riemensperger et al. 2005).

Together, these studies describe training-dependent increases in odor-driven activity in AL (Yu et al. 2004), in DPM neurons (Yu et al. 2005), and in the dopaminergic neurons that contact the MB lobes (Riemensperger et al. 2005). In each of these studies, the increases in odor-driven activity are observed in animals that first were exposed to odors paired with shock. These observations of associative activity changes in the dopaminergic input and DPM modulatory neurons are surprising because they suggest a broader circuitry and invoke the idea of a feedback loop from MB outputs onto the input neurons. But it should be stressed that imaging studies must be integrated with findings from genetic and behavioral dissection of memory. Otherwise, we do not know the phenotypic relevance of the observed phenomenology. For example, all three of the above experiments document an altered odor-evoked activity. Memory consolidation, in contrast, is set in motion by the learning experience and can occur without subsequent odor exposure. The observed increases in neural activity can therefore be reasonably thought of as neural correlates of the conditioned response. As such, they do not identify sites of memory storage per se, but instead may identify part of the circuitry that is activated during memory retrieval. Yet there is direct experimental evidence that activity in DPM and dopaminergic neurons is dispensable for retrieval (Schwaerzel et al. 2003; Keene et al. 2004, 2006; Yu et al. 2005)! What then is the behavioral relevance of the associative odor-evoked activity? It is tempting to speculate that the observed effects could have more

to do with extinction or memory reconsolidation than with consolidation or retrieval per se. These studies nevertheless provide a clear demonstration of the potential for functional imaging to provide a new and highly informative level of analysis to this system.

WHERE DO WE GO FROM HERE?

In this final section of this chapter, we attempt to peer into the future to suggest where we need to go as a field. We propose that three major obstacles must be solved. First, we need to progress beyond identification of genes involved with the biology of memory to an understanding of how networks of genes interact to produce the phenotype of memory. Without more holistic understanding of gene interaction, the impressively long list of genes is not particularly informative. If one looks at the history of genetics, this synthetic outlook typically has been accomplished by the use of modifier screens. By identifying suppressors and enhancers of a given mutation, one finds informative gene interactions that help to put groups of genes into signaling pathways. In general, however, modifier screens are impractical for complex quantitative phenotypes requiring methodical testing of multiple animals. Two possible approaches come to mind. Unlike single-gene mutagenesis, microarray approaches can provide a snapshot of the genomic response (Dubnau et al. 2003b). This method is fraught with signal-to-noise problems, however, and requires significant in vivo follow-up. A second approach that is gaining practicality is selective breeding for phenotypic extremes, which are less labor-intensive than forward mutagenesis for suppressors or enhancers. Like a modifier screen, which identifies pair-wise gene interaction, this approach capitalizes on gene interactions, but it can reveal more complex networks of interaction. The traditional disadvantage of this approach is the difficulty of identifying the loci involved. This technical hurdle is now being overcome in species where the genome is sequenced, where multiple alleles are available, and where micorarray approaches are standard (Anholt and Mackay 2004; Greenspan 2004).

A second obstacle to progress is the complexity of neural circuitry. To understand gene function and neural circuit function, we will need not only to identify all of the genes and characterize all of the relevant neural circuitry, but also to know which genes are acting in which neurons and for which features of the phenotype, e.g., for which memory phase. A good example is the rescue of *rutabaga* STM defect with MB expression (Zars et al. 2000a,b). But even here, we do not know whether MB *rutabaga* expression would be sufficient to give normal LTM. The

tools are available in both the worm and the fly to accomplish this goal for each of the genes identified.

Finally, the greatest weakness of worms and flies both has traditionally been the challenge of doing electrophysiological experiments. We predict that recent advances in both in vivo recording methods (Wilson et al. 2004; O'Hagan et al. 2005) and functional imaging approaches (Suzuki et al. 2003; Wang et al. 2004; Yu et al. 2004, 2005; Riemensperger et al. 2005; Frokjaer-Jensen et al. 2006) will bring to fruition a new level of analysis for these established powerhouses of genetic tinkering.

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